University of Alberta
Printing Department

THE UNIVERSITY OF ALBERTA

A STUDY OF THE BINDING OF INORGANIC IODIDE TO THE PLASMA PROTEINS OF SOME FRESHWATER TELEOST FISHES

BY

CHAU-TING HUANG

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF ZOOLOGY

EDMONTON, ALBERTA

APRIL, 1967

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "A Study of the Binding of Inorganic Iodide to the Plasma Proteins of Some Freshwater Teleost Fishes" submitted by Chau-ting Huang, in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

Of eight non-migratory freshwater teleosts studied, it was found that a significant binding of inorganic iodide in the plasma existed only in species of the Order Clupeiformes including *Esox lucius*, *Coregonus clupeaformis*, *Salmo gairdneri*, and *Thymallus arcticus*. Iodide binding was not found in four other species: *Catostomus commersonii* (Order Cypriniformis), *Perca flavescens* (Order Perciformis), *Stizostedion vitreum* (Order Perciformis), and *Lota lota* (Order Gadiformes). *E. lucius* and *C. clupeaformis* showed a small seasonal variation in binding capacity. The plasma of male fish binds significantly more iodide than does the plasma of female fish.

Inorganic iodide was found to bind with the plasma albumin-like protein. The actual binding sites were presumably the free cationic groups of basic amino acid residues on the protein molecules. Iodide binding was inhibited by NO_3^-, SCN^-, ClO_4^- and CCl_3COO^-, but not affected by thiourea, thiouracil and other halide ions. Binding capacity was increased as the temperature was decreased, but the binding energy was temperature independent. The binding energy was weak in both *E. lucius* (-5.897 KCal/mole at 4°C) and *C. clupeaformis* (-6.252 KCal/mole at 4°C).

ACKNOWLEDGEMENTS

I wish to express my deepest appreciation to Dr. C. P. Hickman, Jr. for his guidance and encouragement throughout this study. I also thank Dr. D. D. Beatty, Dr. R. F. Ruth, and Dr. D. J. Campbell for their critical reviews of the manuscript.

I am also indebted to my fellow graduate students, Mr. Alfred J. Wiggs and Mr. William C. Mackay for much practical advice.

Financial support was provided by the National Research Council to Dr. C. P. Hickman, Jr.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

The strong tendency of proteins to form tightly bound complexes with a variety of ions was noted by Sørensen (1909) early in this century. Later, Klotz, Walker and Pivan (1946) published a series of papers dealing with the interaction of organic anions with dissolved proteins. Scatchard and Black (1949) studied the binding of small monovalent anions to serum albumin. With the recent activity in this field of study, many valuable review articles concerning both experimental methods and theoretical analyses have appeared (Klotz, 1950, 1953; Gurd and Wilcox, 1956; Vallee, 1955; Rosenberg and Klotz, 1960; Scatchard *et al.*, 1954). In general, the ion-protein interaction is non-specific in nature, but the binding affinity is related to the protein structure and the nature of the binding ion, (Foster, 1960).

Many substances are bound to the blood proteins. Among these substances, much interest has centered on the interaction of plasma proteins with the thyroid hormones. The thyroxine-binding serum proteins have been found in different species of vertebrates (Robbins and Rall, 1960). In man, for example, thyroxine is bound to the α-globulin (TBG), albumin and prealbumin (TBPA) (Ingbar and Freinkel, 1960; Robbins and Rall, 1957, 1960; Sterling and Tabachnick, 1961). Sterling, Rosen and Tabachnick (1962) developed the concept that binding is universally based on an interaction of anionic phenolate groups of thyroxine with the cationic groups on the albumin protein molecules.

In addition to the binding of hormonal organic iodine to the plasma proteins, a characteristic evidently present in all vertebrates, Leloup and Fontaine (1960) discovered a significant binding of inorganic iodide to the plasma protein of teleost fishes. This binding characteristic appeared to be restricted to a few species of teleosts and was not found in other vertebrates. The binding capacity was associated with the physiological status of the fish, especially with the migratory behavior and ovary maturation. Leloup (1964b) found that the inorganic iodide was bound only to the plasma albumin fraction of the plasma proteins.

The purpose of this research was to (1) confirm the work of Leloup and Fontaine (1960) and Leloup (1964b) on the nature of the iodide-protein interaction, (2) additionally study for the first time the influence of sex, season, pH and protein acetylation on the iodide binding capacity of the protein, and (3) to determine the distribution of the iodide binding characteristic among the local fishes of Alberta. The physiological importance of this binding characteristic and the binding energy are also considered.

MATERIALS AND METHODS

A. Living Materials

Six species of freshwater teleosts were collected from June, 1965, to November, 1966, from Lac Ste. Anne, a large eutrophic lake northwest of Edmonton, Alberta: northern pike, *Esox lucius*; lake whitefish, *Coregonus clupeaformis*; white sucker, *Catostomus commersonii*; yellow perch, *Perca flavescens*; yellow walleye, *Stizostedion vitreum*; and burbot, *Lota lota*. Arctic grayling, *Thymallus arcticus*, were collected by Dr. D. D. Beatty from Kinky Lake, Alberta, in August, 1965. All fish were caught by gillnet or trap net. Rainbow trout, *Salmo gairdneri*, were obtained from the province of Alberta fish hatchery in Calgary, Alberta, on July 6, 1966. The rainbow trout were held in running dechlorinated water at about 4°C. At the time of sampling, total body weight, gonad weight, fork length and sex were recorded. Scales were collected for age determination. Lac Ste. Anne water temperatures were recorded throughout the period of study (May, 1965, to December, 1966).

B. Blood Sampling

Blood samples were taken at the time fish were collected from the lake. The fish was stunned by a blow to the head, and about 5 cc of blood was withdrawn from the caudal vein with a 10 cc heparinized syringe and 18 gauge needle. The blood was separated immediately by centrifugation in a Buchler centrifuge and the plasma was pipetted into a 15 cc test tube. The blood sampling procedure for *Salmo gairdneri*

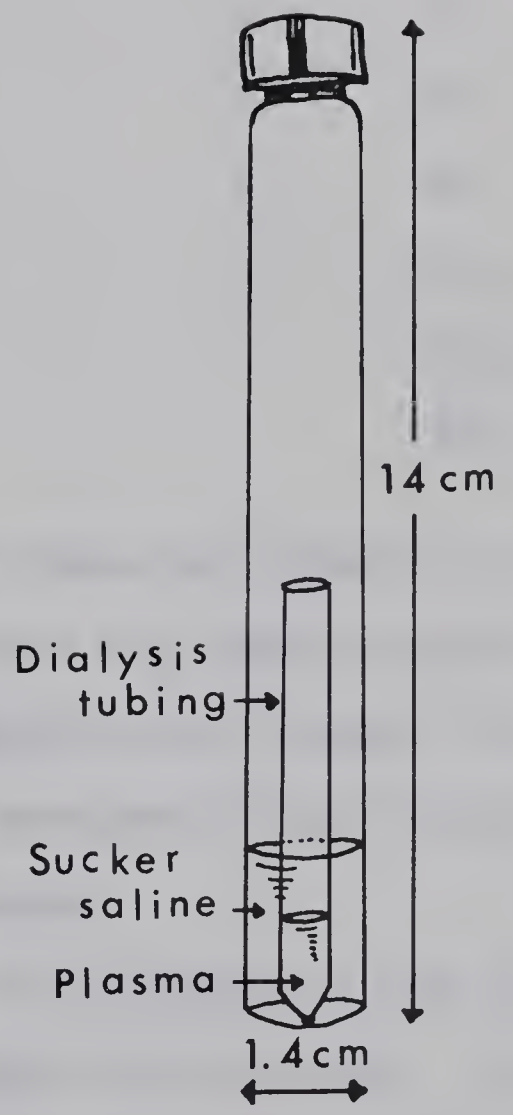
was the same as for fish collected from Lac Ste. Anne, except that a smaller (22 guage) needle was used and the blood was separated in a Beckman Microfuge (Model 152). The separated plasma was frozen immediately on dry ice, transported to the University, and stored in a freezer until analysis.

C. Equilibrium Dialysis of Plasma

The inorganic iodide binding capacity in normal plasma of the eight species of freshwater teleosts listed above was studied by the method of dialysis equilibrium. This is the most commonly employed technique for determining the equilibrium in solution between free ions and those bound to proteins (Klotz, Walker, and Pivan, 1946).

Dialysis tubing made by Union Carbide, size 8/32, average pore radius 24Å, was used. This "Visking" dialysis tubing contained cellulose, glycerine, water and a small amount of sulfur. Tubing was permeable to water and would allow diffusion of low molecular weight compounds in aqueous solution while refusing passage to higher molecular weight materials, such as proteins and bacteria. Prior to use each tubing was rinsed with deionized water and damp dried. A knot was tied into one end, leaving an open portion of tubing about 6 cm in length. 0.25 ml of plasma was pipetted into the tubing, which was then put into a test tube 14 cm long x 1.4 cm in diameter (see Fig. 1) containing 3 ml of freshwater fish saline and 0.005 ml of $Na^{125}I$ solution (1.5 µc). The fish saline was formulated from the measured composition

Figure 1. Arrangement for equilibrium dialysis of teleost plasma against isotonic fish saline. 0.25 ml plasma was placed in the dialysis tubing and 3 ml fish saline containing 0.005 ml $Na^{125}I$ (1.5 µc) was added to the test tube.


14 cm
Dialysis tubing
Sucker saline
Plasma
1.4cm

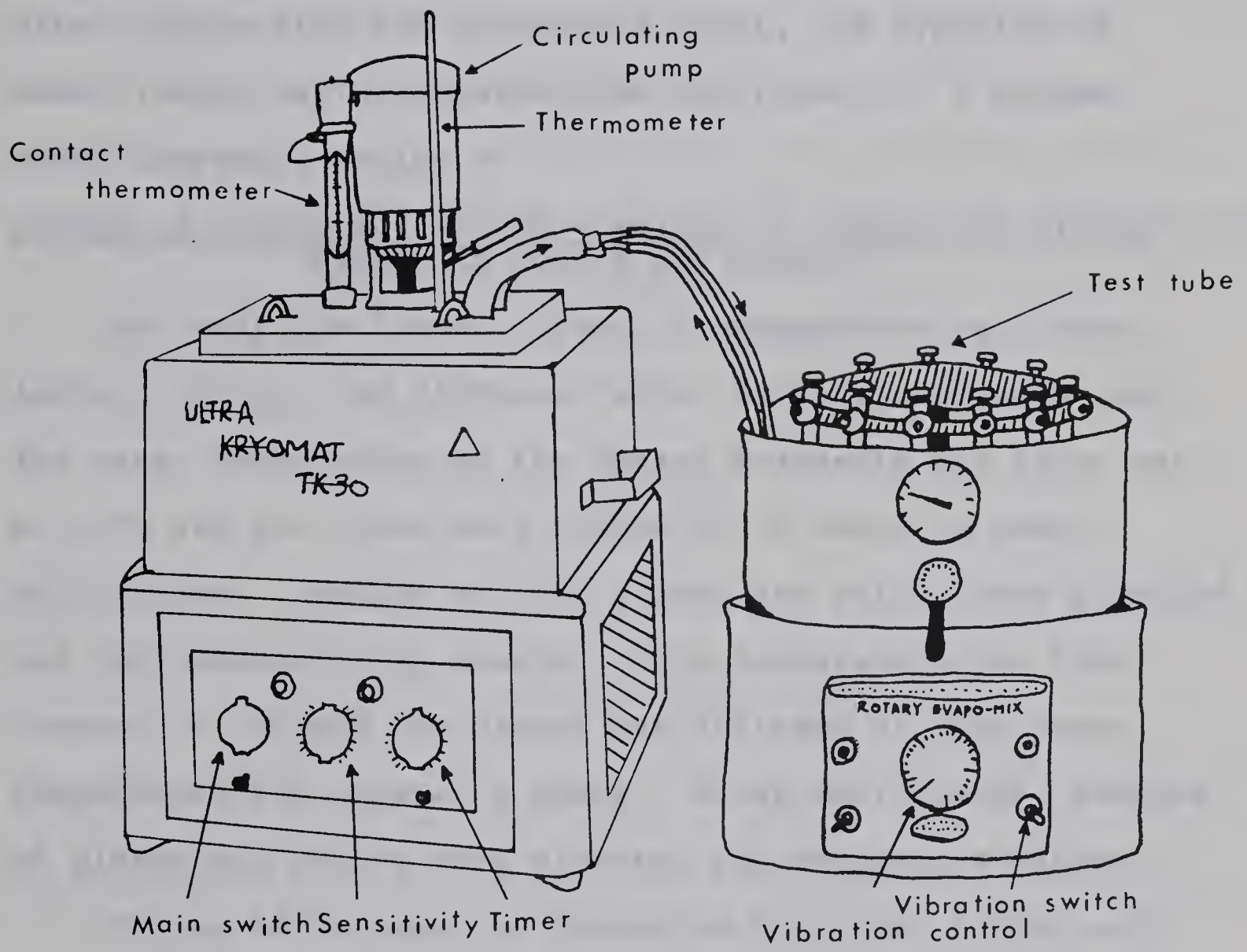
of white sucker plasma by Dr. C. P. Hickman for use in this laboratory. The saline had the following composition.

Chemical	Concentration (%)	Parts Used	Ion	Concentration (mM/liter)
NaCl	0.935	95	Na	147
KCl	1.02	4	Cl	155
$NaHCO_3$	1.35	3	K	7
$CaCl_2$	1.49	3	Ca	15
KH_2PO_4	2.19	1	Mg	7
$MgSO_4$	2.40	1	SO_4	8
			HCO_3	4.6
			PO_4	2

Because the physical-chemical properties of the radio-isotope iodine-125 were the same as stable iodine-127, its radioactivity was applied as a tracer for this study. The $Na^{125}I$ solution was purchased from Atomic Energy of Canada Limited, Ottawa, Canada.

Each test tube was clamped to the vibrating plate of a Buchler Rotary Evapo-Mix apparatus. The bottom quarter of the tube was under water. The water temperature was controlled by a Lauda Ultra Kryomate TK-30 which provided a temperature constancy of $\pm$0.2°C (Fig. 2). Plasma was dialysed against the isotonic sucker saline at 20°C. Six hours was found adequate to achieve equilibrium, but longer shaking was usually employed. After equilibrium, 0.01 ml samples were taken from both the plasma and its dialysing medium using a 0.01 ml Misco calibrated micropipette. Samples

Figure 2. Mechanical apparatus for equilibrium dialysis. The dialysis test tube was held on the Rotary Evapo-Mix. It contained a water bath and rotation control. The shaking action speeded the dialysis process. The Ultra Kryomat TK-30 controlled the water temperature of the Rotary Evaop-Mix.


Circulating pump
Thermometer
Contact thermometer
Test tube
ULTRA KRYOMAT TK30
ROTARY EVAPO-MIX
Main switch
Sensitivity
Timer
Vibration switch
Vibration control

were transferred to separate Nuclear-Chicago Well Scintillation counting tubes (11.2 cm in length and 1.6 cm in diameter) containing 3 ml of distilled water. The solution was mixed and counted in a Nuclear-Chicago Ultrascaler II Well-type Scintillation Counter for 10 minutes or to 40,000 counts. After subtracting the background count, the fraction of bound iodide was calculated from the formula: % plasma-bound inorganic iodide =

$$\frac{\text{plasma in counts per minute - saline in counts per minute}}{\text{plasma in counts per minute}} \times 100 \quad (1)$$

To study the direct effect of temperature on plasma iodide binding, two different water temperatures were used. The water temperature of the Rotary Evapo-Mix was first set at 20°C and the tubes were shaken for 6 hours to reach equilibrium. Samples of both plasma and saline were pipetted and the radioactivity counted. The temperature was then lowered to 4°C and the plasma was dialysed at this lower temperature for another 6 hours. After equilibrium, samples of plasma and saline were pipetted and counted as before.

It was of interest to determine how other anions and cations affected the iodide-binding behavior of the plasma proteins. For this purpose, solutions of NaF, NaBr, NaCl, $NaNO_3$, NaSCN, $NaClO_4$, and NaI were added separately to the plasma of *E. lucius*, *C. clupeaformis* and *S. gairdneri* to make the final concentration of the added compounds 1×10^{-3}M. In other experiments thiourea and thiouracil were added to plasma to give a final concentration of 1×10^{-3}M for thiourea and 1×10^{-4}M for thiouracil. Dialysis was carried out at

20°C as already described for normal plasma.

The association constant of plasma protein-bound inorganic iodide and the total concentration of the iodide binding sites were determined by a method similar to that used by Booth, *et al*. (1961).

Let ( ) = molar concentration; K = the association constant of plasma protein-bound inorganic iodide; PI: occupied binding sites on the plasma protein=bound inorganic iodide; P = unoccupied binding sites on the plasma protein; I = free (unbound) inorganic iodide; P^t = total binding sites on the plasma protein; I^t = total inorganic iodide. From the law of mass action,

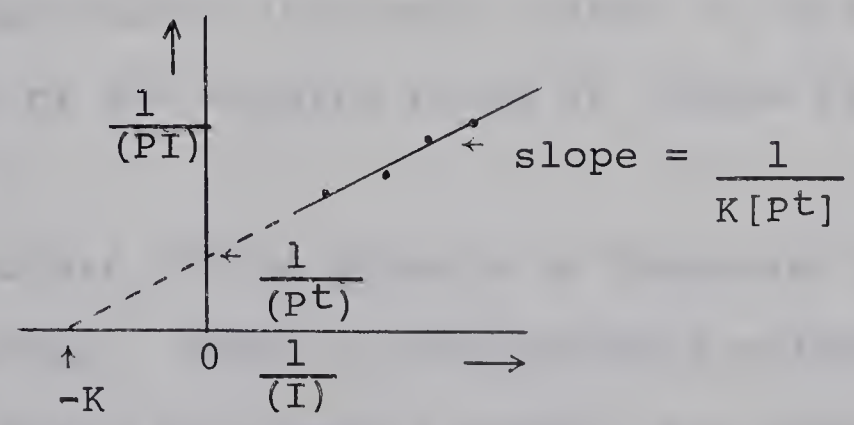
$$K = \frac{(PI)}{(P)\ (I)} \quad - - - - - - - - - (2)$$

and $$(P) = (P^t) - (PI) \quad - - - - - - - - - (3)$$

therefore $$K = \frac{(PI)}{[(P^t) - (PI)]\ (I)}$$

On rearranging $$\frac{1}{(PI)} = \frac{1}{K(P^t)(I)} + \frac{1}{(P^t)} \quad - - - - - - - - - (4)$$

In a graph of $\frac{1}{(PI)}$ versus $\frac{1}{(I)}$, the intercept on the ordinate is $\frac{1}{(P^t)}$ and K is on the abscissa (see diagram below).



The plasma inorganic iodine of *E. lucius* and *C. clupeaformis* was determined by chemical analysis of the protein precipitate washes (Acland, 1957).

For each species of fish, five 0.25 ml plasma samples were pipetted into separate dialysis tubings and prepared for dialysis equilibrium as already described. To each of the 4 tubes was added 0.01 ml of NaI solution with concentrations of 0.0267 M, 0.02 M, 0.0133 M, and 0.0067 M respectively. The fifth served as a control. Each sample was dialysed first at 20°C and then at 4°C. The radioactivity of samples from plasma and saline were measured by the procedures already described. From the following equations:

$$\% \text{ protein-bound inorganic iodide} = \frac{(PI)}{(PI)+(I)} \quad - - - (5)$$

$$\text{and } (I^t) = (PI) + 13\,(I) \quad - - - - - - - - - - - - (6)$$

(I^t) was the total concentration of inorganic iodide in the system (total plasma inorganic iodide plus added stable iodide). Since volume change during dialysis was negligible, the volume of the dialysing saline was 12 times that of the plasma within the tubing. From the above formulas, (PI) and (I) were calculated in each experimental condition, and their reciprocals $\frac{1}{(PI)}$ and $\frac{1}{(I)}$, were plotted as shown above. By regression analysis, a straight line was drawn through these points. The extrapolation gave the value of the association constant of plasma protein-bound inorganic iodide, K, and the total concentration of the binding sites of iodide in the plasma protein (P^t).

The molecular structure of the protein is important in the ion-protein interaction. There is considerable evidence that thyroxine and the anion dye, methyl orange, are combined to free cationic groups of bovine or human plasma albumin (Klotz, *et al.*, 1946; Sterling, Rosen and Tabachnick, 1962).

To study the effect of acid strength on the anion-cation interaction, four kinds of buffers were used to change the pH of plasma from its normal physiological value. The buffers were: sodium acetate pH 4.2 and 5.0; potassium phosphate pH 6.0 and 7.0; sodium barbital pH 8.0 and 9.0; carbonate pH 10.0 and 10.8. All were prepared at 0.2 ionic strength and pH value at 25°C according to the formulas given in Long's Biochemists Handbook (1961). For each buffer, 0.02 ml plasma of *E. lucius* was diluted to 0.25 ml with the buffer. The well mixed plasma solution was found to have the same pH as the buffer used. Procedures for dialysis equilibrium and sample counting were the same as described before except that the appropriate buffer was used as dialyser and the water temperature was 25°C rather than 20°C during dialysis. Finally, small samples of the plasma solution (about 0.005 ml) were applied to an acetate membrane for microzone electrophoresis in barbital buffer, pH 8.6, ionic strength 0.05, applied current 3.0 ma and duration 30 minutes at room temperature. The procedure for microzone electrophoresis will be described in more detail in the next section.

To study the mechanism of binding, acetylated plasma proteins preparations were made. This technique was applied specifically to acetylate and ε-amino groups of lysine residue of the protein under certain special circumstances. Acetylation was done following the procedures of Fraenkel-Conrat, Bean and Lineweaver (1949) and Sterling, *et al.* (1962). 0.5 ml plasma of *E. lucius*, *C. clupeaformis*, and *S. gairdneri* were pipetted separately into 15 ml test tubes containing 0.5 ml

of saturated sodium acetate solution. The plasma solutions were held in a bath of ice and salt at about -5°C. 1.2 ml of acetic anhydride solution was added dropwise over a one hour period to each plasma solution. The mixtures were shaken constantly during the acetylation. The acetylated plasma proteins were precipitated at pH 4.0 by adding 1 ml 0.1 N HCl dropwise and separated by centrifugation. The precipitated proteins were redissolved with 0.25 ml of phosphate buffer, pH 7.0 and ionic strength 0.2. The acetylated protein solutions were put in Visking dialysis tubing. Both ends were knotted and the solutions were dialyzed against daily changes of distilled water for 3 days at 4°C. Finally, 0.1 ml of the acetylated protein solutions were diluted with an equal volume of phosphate buffer, pH 7.0, ionic strength 0.2 and dialysed to equilibrium against this buffer at 25°C.

D. Microzone electrophoresis

The electrophoresis technique is based on the differential mobility of protein molecules in an electric field. In solution, protein is amphoteric in nature, the total net charges on the surface depending on the amino acid content and the pH of the solution. The total net surface charges of a protein are, in turn, associated with its mobility in an electric field. Bovine plasma albumin moved more rapidly because it has more net negative charges on the surface. As a result, it will move faster than larger proteins toward the anode. Microzone electrophoresis has many advantages over other methods. It yields excellent results in protein

separation by using a cellulose acetate support medium. This membrane is formed of a sponge-like cellulose acetate material 120 μ thick, with uniform, interconnected, spherical spaces less than 2 μ in diameter. The membrane is very porous, permitting a free but controlled flow of buffer solution throughout the interconnected spaces. Unlike filter paper, the cellulose acetate does not absorb protein, so that "tailing" after the moving fractions is negligible. In the present study, this method was used to identify the plasma protein fraction which bound inorganic iodide, and to determine the relative amounts of the binding protein in the total plasma protein concentration.

1. *Normal plasma protein separation*

The Beckman microzone electrophoresis cell model R-101 was employed for separation of the plasma proteins. Barbital buffer of pH 8.6 and ionic strength 0.05 was prepared from 1.84 grams barbital and 10.30 grams sodium barbital, made up to one liter with distilled water. The buffer solution was stored in a refrigerator before using. The electrophoresis cell was filled with the buffer to the marked level. The microzone acetate membrane was rinsed with the buffer and the excess buffer removed. The membrane was set horizontally on the top of the electrophoresis cell with the ends of membrane lying in the buffer. Eight different plasma samples were applied to the membrane along a single line. A starting electric current of 3.0 ma was applied for 30 minutes at room temperature as described in the manual for the instrument. Following electrophoresis, the membrane was fixed and stained

immediately in a fixative-dye solution for 10 minutes. The fixative-dye solution contained 0.2% by weight of ponceau-S stain, 3.0% by weight of trichloroacetic acid and 3.0% by weight of sulfosalicyclic acid. The TCA solution caused the precipitation of plasma proteins on the membrane and ponceau-S stained the plasma protein red. After this, the membrane was washed with 5% acetic acid solution to remove the excess of fixative-staining solution. It was then cleaned first in 95% alcohol for 1 minute, and finally in a freshly prepared solution of 25% v/v of glacial acetic acid and 75% v/v of 95% ethanol for 5 minutes. The cleaned membrane was flattened on a glass plate and dried in a 35°C oven. The developed membrane was stored in a plastic envelope.

2. *Protein concentration determination*

Differences in staining intensity of the protein fractions on the developed microzone membrane were proportional to differences in protein concentrations. The membranes were scanned with a Beckman protein Analytrol (model RB), to determine the proportional amounts of each protein fraction (Fig. 3). The total plasma protein concentration was determined by TS-refractometer at room temperature, and the concentration of the albumin-like fraction was calculated as grams per 100 ml of plasma by the following equation:

$$\text{Albumin-like protein (g\%)} = \text{Total plasma protein (g\%)} \times \frac{\text{Albumin-like protein (\%)}}{100} \quad \text{- - - - - - (7)}$$

Figure 3. Relative distribution of plasma protein fractions in lake whitefish, *Coregonus clupeaformis*.

SPINCO DIVISION OF BECKMAN INSTRUMENTS, INC., PALO ALTO, CALIFORNIA MADE IN U.S.A.

WHEN REORDERING SPECIFY CHART 344-319742

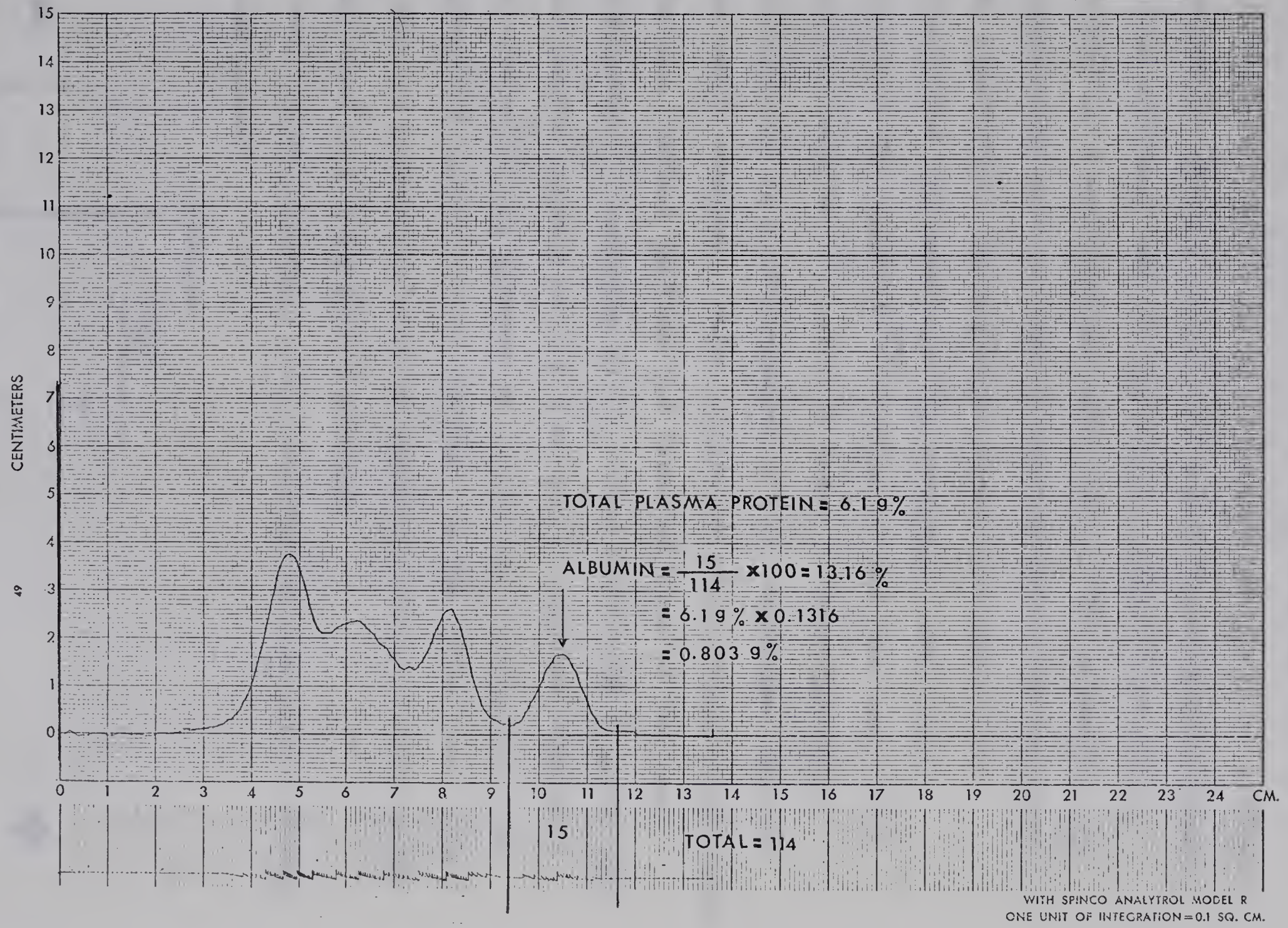
REMARKS:

FILTERS _____ RUN NO. _____

SLIT WIDTH _____ STRIP NO. _____

CAM NO. _____ DATE 20/12 66

SAMPLE W 121

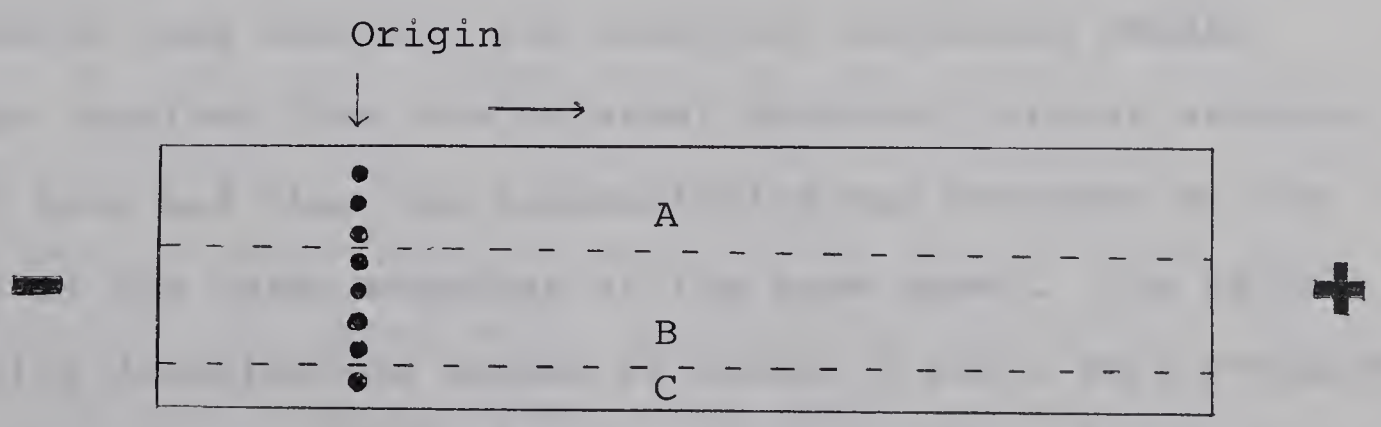


WITH SPINCO ANALYTROL MODEL R
ONE UNIT OF INTEGRATION = 0.1 SQ. CM.

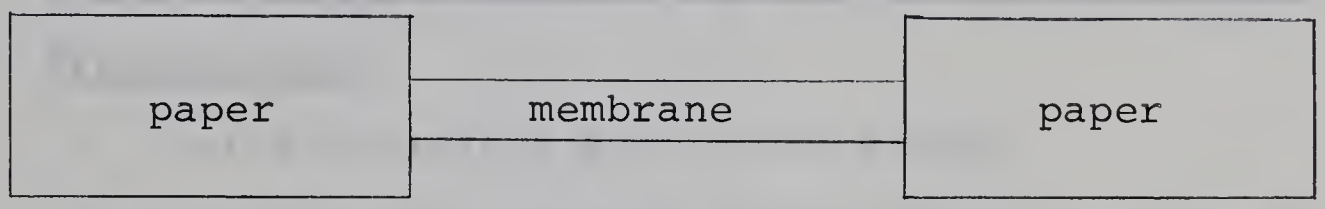
3. *Separation of plasma proteins containing* $Na^{125}I$

0.005 ml of $Na^{125}I$ (2 μc) solution was added to 0.02 ml of freshwater teleost plasma (all those listed before except *Thymallus arcticus*) and bovine plasma, mixed thoroughly, and stored in a refrigerator over night. The procedure for electrophoresis was the same as already described for normal plasma separation. Of the eight places available for application of the sample on the membrane, seven were applied with the plasma-$Na^{125}I$ solution and the last place was applied with $Na^{125}I$ solution alone. Electrophoresis was carried out in a cold room at 4°C to avoid heat effects on the iodide binding. Under different experimental conditions of starting current applied and duration time of the run, the plasma-$Na^{125}I$ solution of *C. clupeaformis* and *S. gairdneri* yielded better results with 2.0 ma for 20 minutes than with either 3.0 ma for 20 minutes or 1.0 ma for 30 minutes. The plasma-$Na^{125}I$ solution of *E. lucius*, on the other hand, gave good protein separation with no apparent effect on the iodide binding even at 3.0 ma for 30 minutes.

After electrophoresis, the membrane was cut into 3 strips as shown below:



Strip A and Strip B contained the plasma-$Na^{125}I$, Strip C contained only the $Na^{125}I$. Strip A was passed through the procedures of fixation, staining, cleaning and drying as described before. Strips B and C were covered with cellulose scotch tape on both sides and the membranes were connected at both ends to long paper strips, 3 x 60 cm as shown in the following diagram, so that they could be scanned with a paper-strip radioactivity scanner system:



Nuclear-Chicago Ratemeter (model 1620CS) was used in the combination with Nuclear-Chicago Scintillation Detector DS 8, Nuclear-Chicago Actigraph Chromatogram Scanner (model C-100B) and Nuclear-Chicago Recorder (model R1000). Radioactivity on the membrane was received first by the detector as the paper-membrane strip was scanned through the narrow window (to 1/16 inch wide) of the Actigraph at a speed of 0.75 inch/hour. The Ratemeter, by using the 40 second time constant for counting, converted random pulses received from the external detector into an average count rate and then the radioactivity was recorded on the chart of the outer recorder at the same speed. The radioactivity distribution curves of Strips B and C were compared with the plasma protein fraction distribution of Strip A. Two radioactive peaks were present on Strip B: one sharp

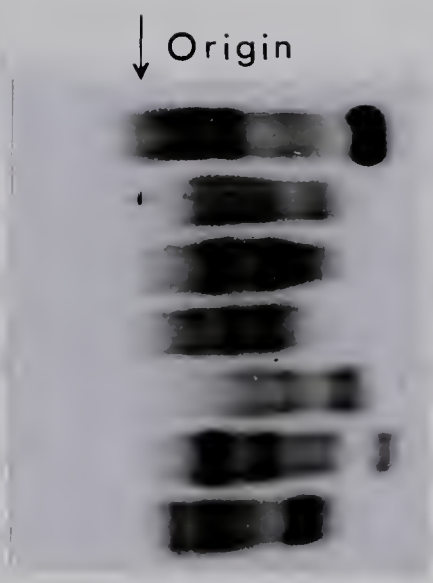
peak was located on the farthest moving protein fraction; the other smaller peak was located remote from the protein fractions toward the anode of the electric field. The smaller peak was identified as free iodide-125 when compared with the radioactivity distribution of Strip C (Fig. 13). The farthest moving fractions of the plasma protein of *E. lucius*, *C. clupeaformis*, and *S. gairdneri* have mobilities similar to bovine plasma albumin (Fig. 4).

E. Iodide Binding Properties of Plasma Proteins After Precipitation

1. *Trichloroacetic acid (TCA) method*

Scatchard and co-workers (1957) found that the binding affinity of trichloroacetic acid to plasma protein was higher than iodide in an electrode system involving an anion exchange membrane. Leloup and Fontaine (1960) stated that plasma iodide binding in some teleosts was destroyed by trichloroacetic acid precipitation of plasma proteins. In the present study, the precipitation method used followed the procedure of Hickman (1961). 0.005 ml of $Na^{125}I$ (2 µc) was added to 1 ml of plasma of *E. lucius*, *C. clupeaformis* or *S. gairdneri* in a 15 ml test tube and mixed thoroughly. 0.5 ml of each mixed plasma solution was pipetted into a 15 ml centrifuge tube. 2.0 ml of 20% trichloroacetic acid was added, stirred and then centrifuged at 2,500 rpm for 15 minutes. The supernatant was decanted and saved. The precipitate was washed three times with 2 ml of 2.5% trichloroacetic acid solution and all the supernatant washings

Figure 4. Microzone electrophoretic patterns of plasma proteins on cellulose acetate membrane. Barbital buffer pH 8.6 and ionic strength 0.05. Applied current 3.0 ma for 30 minutes. Plasma samples from top to bottom of the membrane: bovine, *Lota lota*, *Stizostedion vitreum*, *Catostomus commersonii*, *Salmo gairdneri*, *Coregonus clupeaformis* and *Exos lucius*.


Origin
Bovine
Lota lota
Stizostedion vitreum
Catostomus commersonii
Salmo gairdneri
Coregonus clupeaformis
Esox lucius
−
+

were combined. The precipitate was dissolved in 0.1 N NaOH and diluted to the same volume as the combined supernatant washings. 0.01 ml of sample was pipetted from both the supernatant washings and dissolved precipitate into counting tubes containing 3 ml of distilled water, mixed well and counted in the Nuclear-Chicago Ultrascaler II Well-Scintillation Counter for 10 minutes or 40,000 counts. From the radioactivity of both fractions: % plasma protein-bound ^{125}I =

$$\frac{\text{precipitate in counts per minute}}{\text{precipitate in counts per minute + supernatant in counts per minute}} \times 100 \quad - - \quad (8)$$

2. *Half-saturated ammonium sulfate method*

This method was used to separate the globulins from the albumin of plasma proteins by precipitating the globulins in half-saturated ammonium sulfate solution. Leloup (1964) noted that the plasma protein-bound inorganic iodide was found in the non-precipitated protein fraction after this precipitation method. In the present study, the experimental procedures for both protein precipitation and radioactivity determination were the same as described in the method above, except that half-saturated ammonium sulfate, $(NH_4)_2SO_4$, solution was used as the precipitating reagent and precipitate washing.

RESULTS

A. Plasma Protein Binding of Inorganic Iodide as Revealed by Equilibrium Dialysis of Plasma

1. *Extent of plasma protein binding of iodide among the fish studied*

Of the eight different species of non-migratory freshwater teleosts investigated in this study, only *E. lucius*, *C. clupeaformis*, *S. gairdneri*, and *T. arcticus* showed plasma protein binding of inorganic iodide by the equilibrium dialysis method (Table I). These four species of fish all belong to the Order Clupeiformes. Leloup (1964) noted that among the vertebrates this characteristic was restricted specifically to the teleosts, and further that it was present only in the Order Clupeiformes and Order Mugiliformes. Leloup pointed out, however, that protein binding of inorganic iodide was found among both marine and freshwater representatives and in all anadromous migratory forms studied.

2. *Seasonal differences in iodide binding*

Seasonal variation in the water temperature of Lac Ste. Anne from surface to bottom was measured during 1965 and 1966 and is shown in Fig. 4. The average 3-meter depth water temperature was taken as representative of the environmental temperature for *E. lucius* and *C. clupeaformis*. The lake water temperature was highest in July (16-20°C) and below 4°C for the period mid-November to mid-April.

Table I. Species distribution of the plasma protein-bound inorganic iodide in non-migratory freshwater teleosts.

Order Name	Family Name	Species Name	Common Name	% Plasma protein-bound inorganic iodide (Equation 1)
Clupeiformes	Esocidea	*Esox lucius*	Northern pike	80.85(20°C), 88.85(4°C)
	Coregonidea	*Coregonus clupeaformis*	Lake white fish	80.28(20°C), 88.75(4°C)
	Salmonidae	*Salmo gairdneri*	Rainbow trout	84.30(20°C), 92.23(4°C)
	Thymallidae	*Thymallus arcticus*	Arctic grayling	80.97(26°C)
Cypriniformes	Catostomidea	*Catostomus commersonii*	White sucker	0
Perciformes	Percidae	*Perca flavescens*	Yellow perch	0
Perciformes	Percidae	*Stizostedion vitreum*	Yellow walleye	0
Gadiformes	Gadidae	*Lota lota*	Ling or Burbot	0

Figure 5 and Figure 6 show the seasonal iodide binding capacity of the plasma proteins and the gonad maturation of *E. lucius* and *C. clupeaformis*. There appeared to be some relationship between ovary maturation and the protein-iodide binding capacity, since *E. lucius* showed a decrease in binding just after spawning and during the summer when little size increase in ovaries occurred. In females but not in males, binding increased in spring just before spawning. In *C. clupeaformis* the binding capacity was higher in late July as the ovary just started to increase in size, then decreased progressively as the ovary became mature in late November. The overall seasonal changes are small but it is significant that both sexes of the two species show similar seasonal variations.

Table II shows that male fish have a significantly higher binding capacity than females ($t = 5.57 > t_{(p=0.01)} = 2.632$ and $t = 4.57 > t_{(p=0.01)} = 2.626$ for *E. lucius* and *C. clupeaformis* respectively). However, the sex difference in binding capacity is probably not directly related to albumin concentration, since in *E. lucius* and *C. clupeaformis* male fish had no significantly higher plasma albumin concentration than females (Table III). It is also noteworthy that there was no significant positive correlation between the percentage plasma-iodide binding and the plasma albumin concentration in either species (Table IV).

Figure 5. Seasonal change in water temperature of Lac Ste. Anne, a large eutrophic lake northwest of Edmonton, Alberta, from which most of the fish were collected.

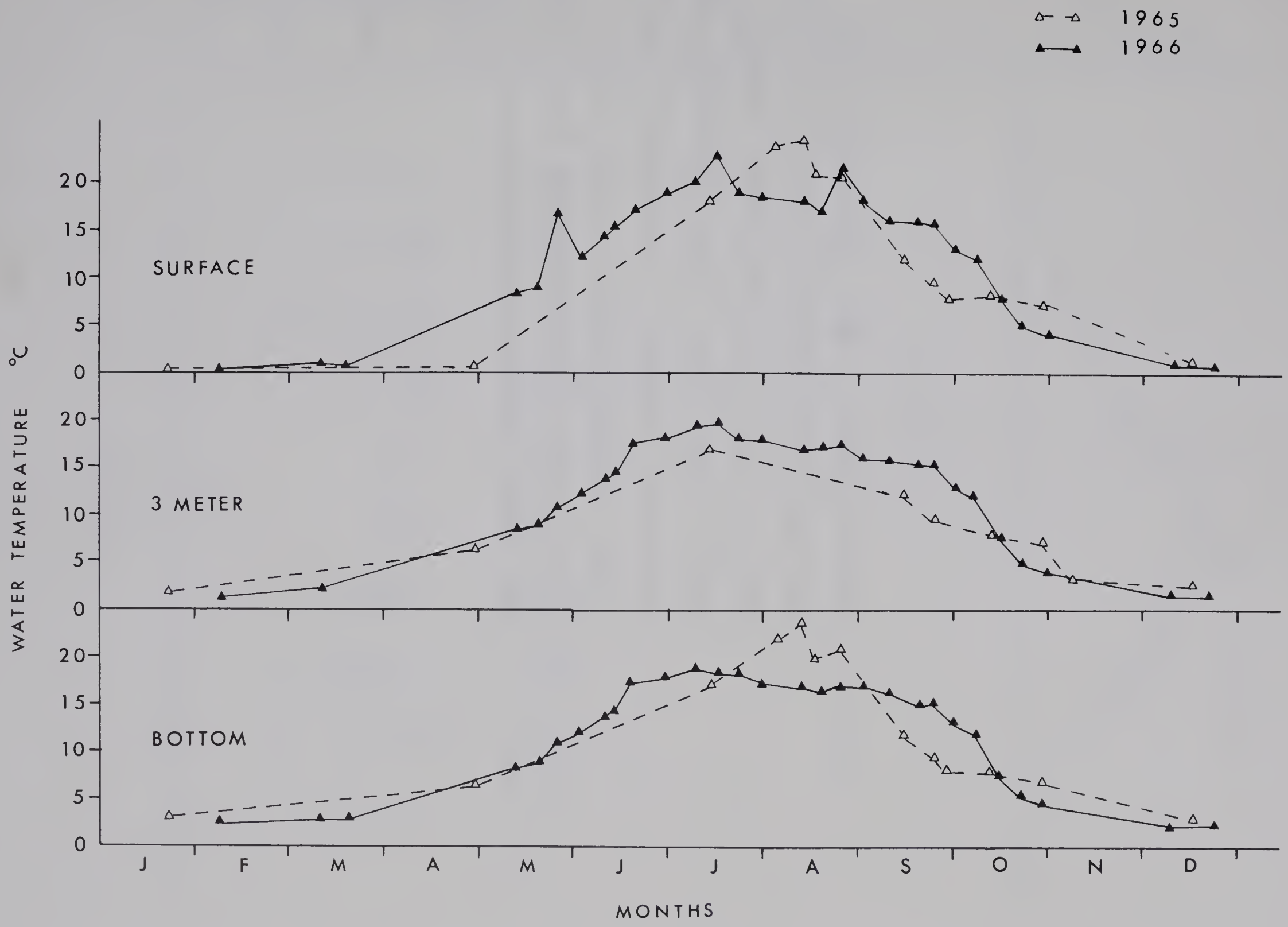

1965
1966
SURFACE
3 METER
BOTTOM
WATER TEMPERATURE °C
20
15
10
5
0
J
F
M
A
M
J
J
A
S
O
N
D
MONTHS

Figure 6. Seasonal change in water temperature of the environment, gonadosomatic index (G.I.), and percentage of plasma protein-bound inorganic iodide of *Esox lucius*. Water temperature was measured at 3 m depth. Gonadosomatic index = (weight of gonads/weight of fish) x 100. Vertical lines indicate standard error of the mean; small numbers indicate sample size.

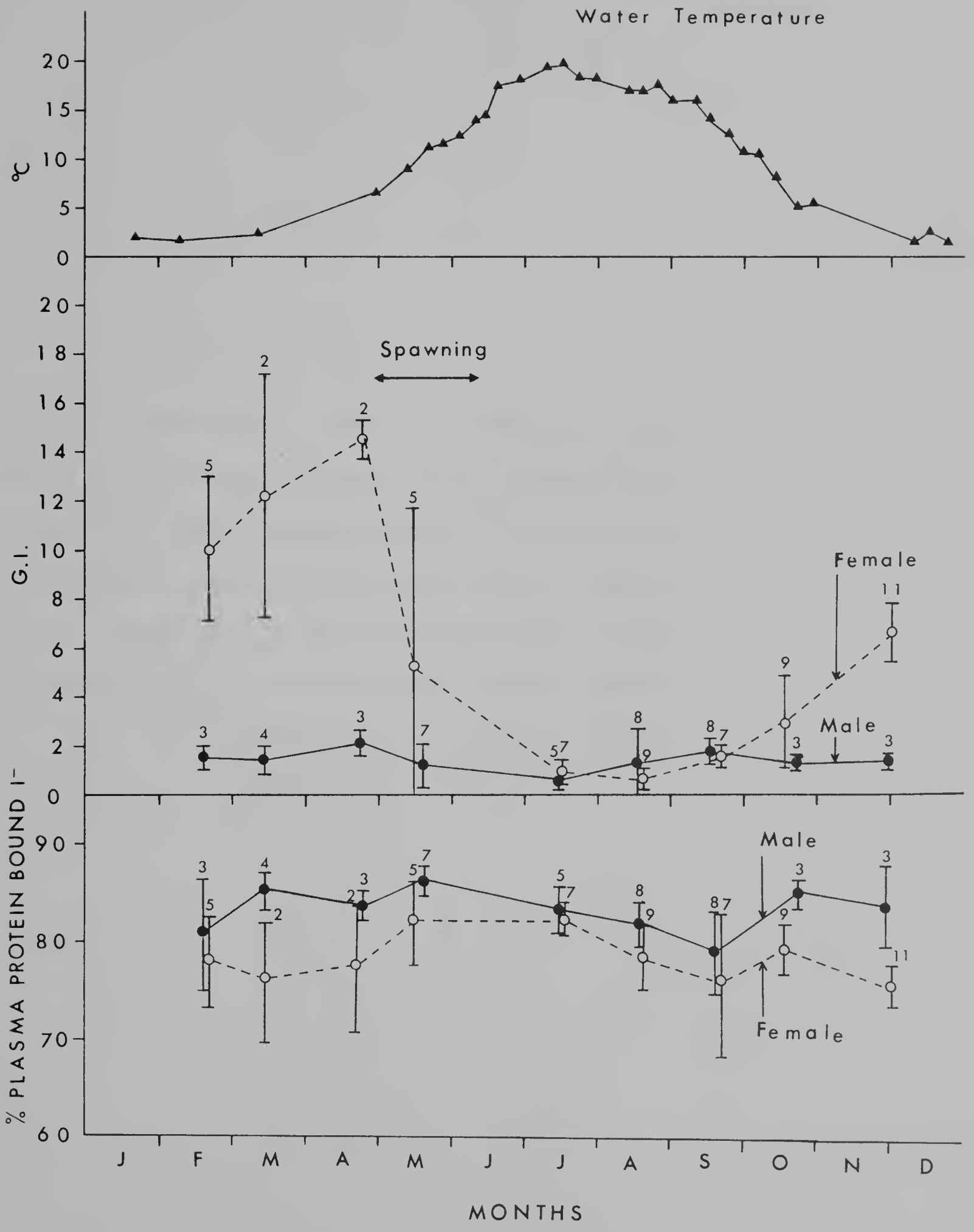
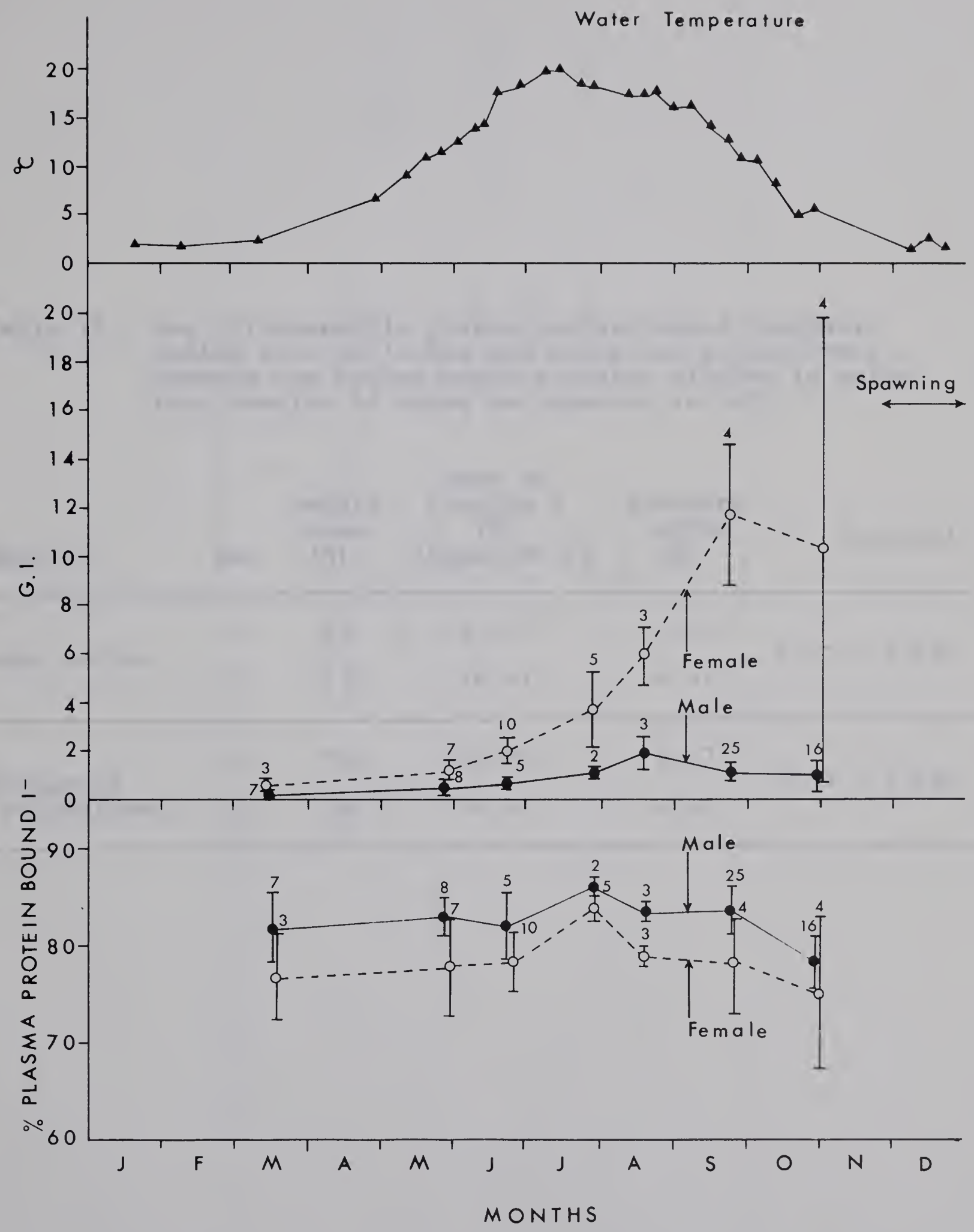

Water Temperature
℃
20
15
10
5
0
Spawning
Female
Male
G.I.
% PLASMA PROTEIN BOUND I⁻
J
F
M
A
M
J
J
A
S
O
N
D
MONTHS

Figure 7. Seasonal change in water temperature of the environment, gonadosomatic index (G.I.), and percentage of plasma protein-bound inorganic iodide of *Coregonus clupeaformis*. Water temperature was measured at 3 m depth. Vertical lines indicate standard error of the mean; small numbers indicate sample size.


Water Temperature
℃
20
15
10
5
0
G.I.
20
18
16
14
12
10
8
6
4
2
0
Spawning
Female
Male
% PLASMA PROTEIN BOUND I–
90
80
70
60
Male
Female
J
F
M
A
M
J
J
A
S
O
N
D
MONTHS

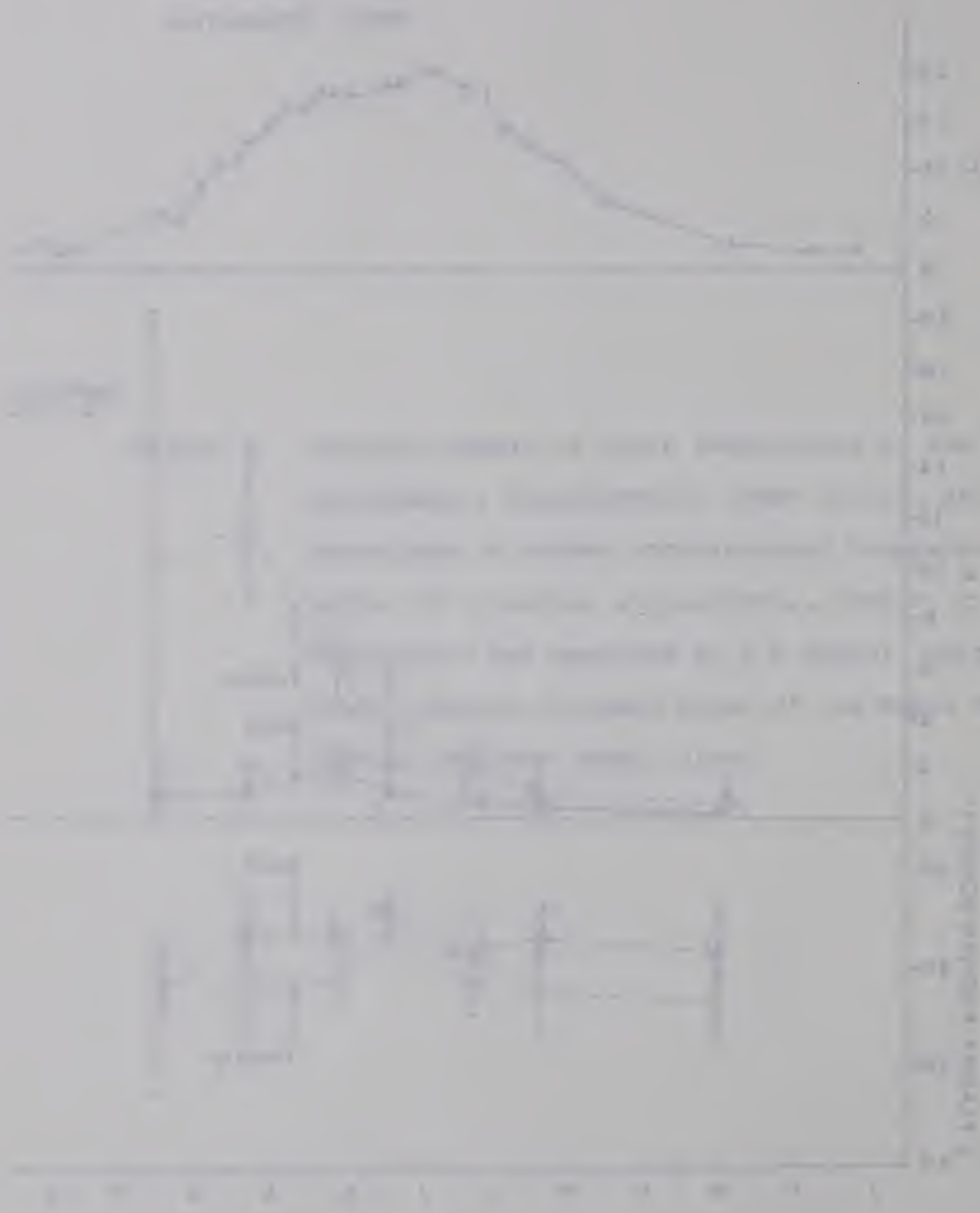


Table II. Sex difference in plasma protein-bound inorganic iodide in *Esox lucius* and *Coregonus clupeaformis*, showing the higher protein-iodide binding in males than females in these two species in 20°C.

Species	Sex	Sample size (N)	Mean of binding % ($\bar{X}$) (Equation 1)	Standard error (S)	t	$t_{(p=0.01)}$
Esox lucius	♂	44	83.13	3.68	5.57	> 2.632
	♀	57	78.54	4.42		
Coregonus clupeaformis	♂	66	82.05	3.37	4.57	> 2.626
	♀	36	78.50	4.68		

Table III. Sex difference and plasma albumin concentration in *Esox lucius* and *Coregonus clupeaformis*.

Species	Sex	Sample size (N)	Mean of albumin concentration (g%) ($\bar{X}$) (Equation 7)	Standard error (S)	t	$t_{(p=0.01)}$
Esox lucius	♂	13	1.998	0.338	2.234	< 2.845
	♀	9	1.674	0.348		
Coregonus clupeaformis	♂	17	0.862	0.221	0.82	< 2.819
	♀	7	0.789	0.105		

Table IV. Test for correlation between plasma-iodide binding percentage and plasma albumin concentration in *Esox lucius* and *Coregonus clupeaformis*.

Species	Sex	Sample size (N)	Correlation coefficient (r)	t	$t_{(p=0.01)}$
Esox lucius	♂	13	0.432	1.589	< 3.106
	♀	9	0.788	3.387	< 3.499
Coregonus clupeaformis	♂	17	0.253	1.014	< 2.947
	♀	7	0.475	1.207	< 4.032

3. *Direct temperature effect*

Two experiments were carried out to study the direct temperature effect on protein-iodide binding affinity. In the first experiment, 11 samples of *E. lucius* and 5 samples of *C. clupeaformis* were dialysed to equilibrium at 20°C and 4°C using the same procedure as for normal plasma dialysis equilibrium. The results are given in Table V. Iodide binding to the plasma proteins of both species was greater at the lower temperature (4°C) than at the higher temperature (20°C), a difference that was statistically significant ($t = 10.50 > t_{(p=0.01)} = 2.845$ and $t = 6.51 > t_{(p=0.01)} = 3.355$ for *E. lucius* and *C. clupeaformis* respectively). In a second experiment the association constant (K) of plasma protein-bound inorganic iodide of *E. lucius* and *C. clupeaformis* was determined at 20°C and 4°C. As shown in Table VI, the association constant was larger at the lower temperature.

4. *Effect of iodide concentration*

Figure 8 shows that as the concentration of $Na^{127}I$ increased, the percentage binding of ^{125}I with the plasma proteins of *E. lucius* and *C. clupeaformis* decreased. Since the radioactive ^{125}I and stable ^{127}I have similar physical-chemical properties, and their proportional distributions are the same, the results indicate increased saturation of the binding sites for iodide. The total iodide binding is actually increased.

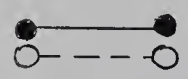
Table V. Direct temperature effect on the plasma protein-bound inorganic iodide in *Esox lucius* and *Coregonus clupeaformis*.

Species	Sex	Sample size (N)	% Plasma protein-bound I^- 20°C (Equation 1)	% Plasma protein-bound I^- 4°C (Equation 1)	t	$t_{(p=0.01)}$
Esox lucius	♀	11	75.64±2.31	85.26±2.0	10.50	> 2.845
Coregonus clupeaformis	♀	5	78.06±3.08	87.46±1.14	6.51	> 3.355

Table VI. The association constant (K) of plasma protein-bound inorganic iodide at 20°C and 4°C, the total inorganic iodide binding sites on the plasma protein (P^t) and the binding energy ($\Delta\mathring{F}$) for plasma of *Esox lucius* and *Coregonus clupeaformis*.

Species	K (liter/mole) (Equation 4)		P^t (mg I^-/liter) (Equation 4)	$-\Delta\mathring{F}$ (Kcal/mole) (Equation 9)	
	20°C	4°C		20°C	4°C
Esox lucius	0.31×10^5	0.43×10^5	12.09	6.054	5.897
Coregonus clupeaformis	0.24×10^5	0.81×10^5	5.76	5.903	6.252

Figure 8. Effect of stable iodide concentration on the plasma protein-bound ^{125}I in *Esox lucius* and *Coregonus clupeaformis*. Experimental temperature 20°C and 4°C.



% plasma protein-bound ^{125}I

Concentration of plasma protein-bound ^{127}I

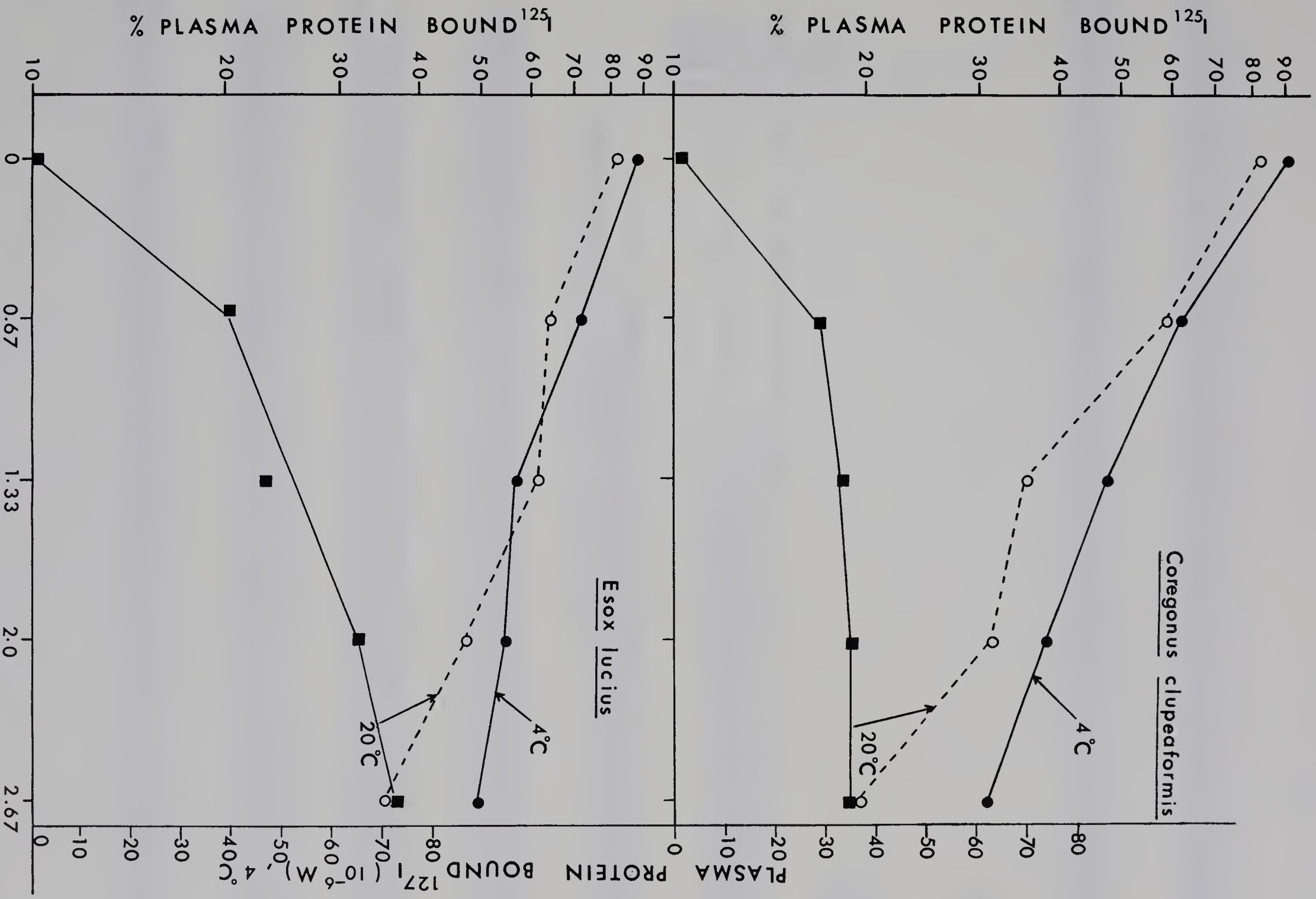
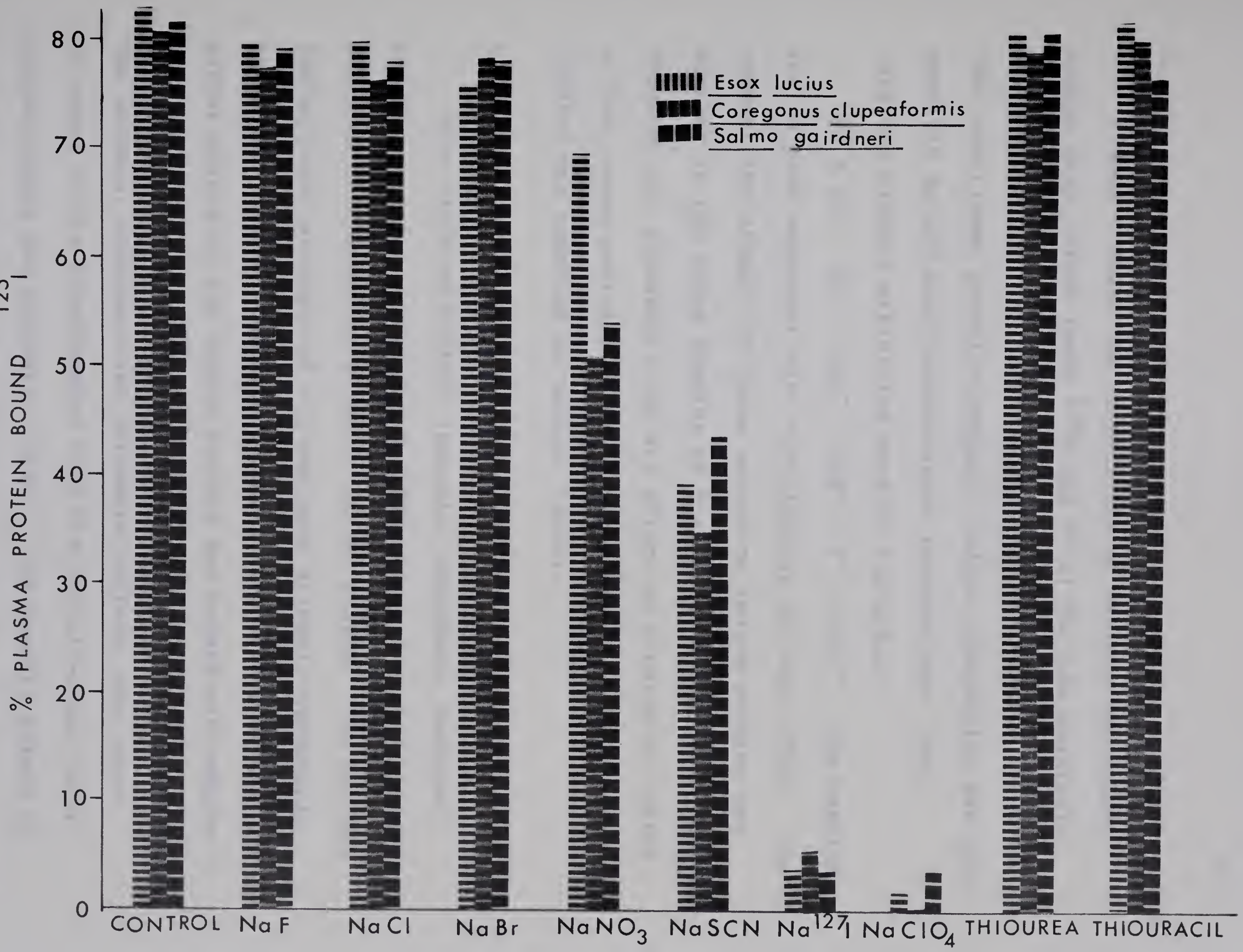

% PLASMA PROTEIN BOUND ^{125}I
10
20
30
40
50
60
70
80
90
0
0.67
1.33
2.0
2.67
ADDED NON-RADIOACTIVE $Na^{127}I$, (10^{-2} M)
PLASMA PROTEIN BOUND ^{127}I (10^{-6} M), 4°C
Coregonus clupeaformis
Esox lucius
4°C
20°C

Figure 9. Effect of monovalent anions and antithyroid substances on the plasma protein-bound inorganic iodide of *Esox lucius*, *Coregonus clupeaformis* and *Salmo gairdneri*. Experimental temperature 20°C.


% PLASMA PROTEIN BOUND ^{125}I
80
70
60
50
40
30
20
10
0
Esox lucius
Coregonus clupeaformis
Salmo gairdneri
CONTROL
Na F
Na Cl
Na Br
Na NO_3
Na SCN
Na ^{127}I
Na ClO_4
THIOUREA
THIOURACIL

5. *Effect of other monovalent anions and antithyroid substances*

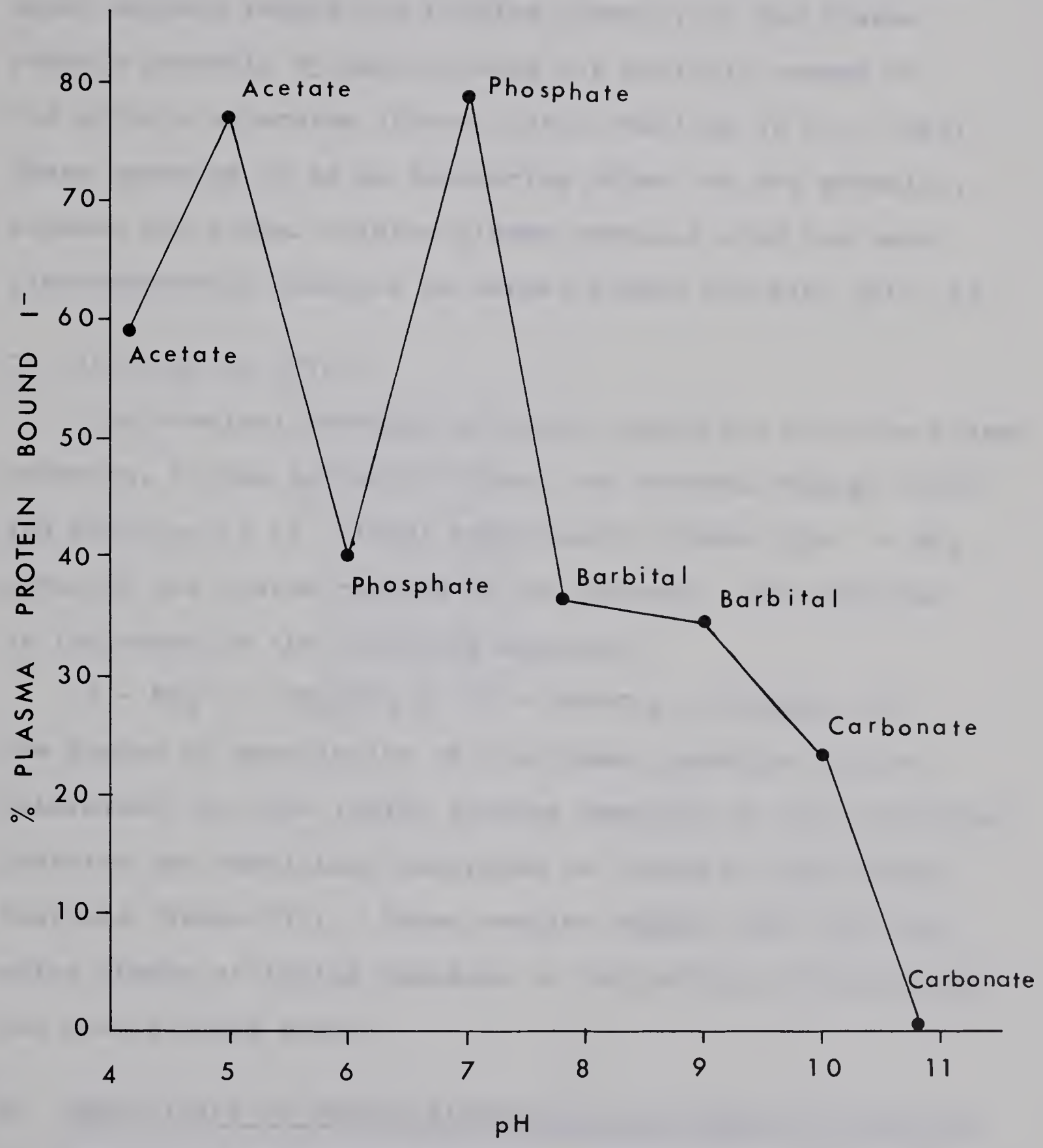
Figure 9 shows the competition of various monovalent anions with iodide (both ^{125}I and ^{127}I) for the available binding sites on the plasma proteins. The results indicate that the plasma protein-inorganic iodide interaction was non-specific as are most protein-anion interactions. The relative binding affinities were of the order:

$F^- = Cl^- < Br^- < NO_3^- < SCN^- < I^- = ClO_4^-$. The results are in good agreement with the findings of Carr (1952). The competitive effects of these anions on iodide binding was similar in the three species of fish studied. However, neither thiourea nor thiouracil had any effect on binding of iodide to the plasma proteins of these three species. This same finding was reported by Leloup (1964b).

6. *Effect of pH and nature of buffers*

Four kinds of buffers (acetate, phosphate, barbital and carbonate) representing 8 different acid strengths were employed to change the pH of *E. lucius* plasma. The buffers had an ionic strength of 0.2 and were slightly hypertonic to normal plasma. The direct competitive effect of the buffer anions on the iodide binding was relatively smaller than their effect on pH, because the buffers used, especially the acetate, phosphate and carbonate buffers, are known to cause little interference with the protein-anion interaction (Klotz and Urquhart, 1949a). The overall effect of pH on the iodide binding affinity is shown in Fig. 10. Iodide binding was least affected at the lower pH's. As the plasma

Figure 10. Effect of pH and buffers on the plasma protein-bound inorganic iodide in *Eosx lucius*. Plasma was diluted 12 times with buffer. Dialysis equilibrium at 25°C.


% PLASMA PROTEIN BOUND I⁻
80
70
60
50
40
30
20
10
0
4
5
6
7
8
9
10
11
pH
Acetate
Acetate
Phosphate
Phosphate
Barbital
Barbital
Carbonate
Carbonate

solution became more basic, the binding capacity dropped significantly and finally completely disappeared at pH 10.8. Basic buffers reduce the binding capacity of the plasma protein probably by neutralizing the cationic groups of the protein molecules (Klotz, 1950; Sterling *et al.*, 1962). There appeared to be no denaturing effect on the proteins, because the buffer diluted plasma proteins show the same electrophoretic behavior as normal plasma proteins (Fig. 11).

7. *Acetylation effect*

The chemical reaction of acetic anhydride with the plasma proteins, by the method of Olcott and Fraenkel-Conrat (1947) and Sterling *et al.* (1962) specifically blocks the ε - NH_3 group of the lysine residue on the protein. The reaction is indicated by the following equation:

$$P - NH_3^+ + (CH_3CO)_2\,0 \rightarrow P - NHCOCH_3 + CH_3CO_2H + H^+$$

The degree of acetylation of the plasma proteins was not determined, but the iodide binding capacity of the acetylated proteins was completely destroyed as judged by equilibrium dialysis (Table VII). These results suggest that the free amino groups of lysine residues in the protein molecule were the main binding sites.

B. Specificity of Iodide Binding by the Plasma Proteins as Revealed by Microzone Electrophoresis

1. *Electrophoretic pattern of normal plasma protein*

Plasma of six species of teleosts (*Esox lucius*, *Coregonus clupeaformis*, *Salmo gairdneri*, *Catostomus commersonii*,

Figure 11. Comparison of microzone electrophoretic patterns of *Esox lucius* buffer diluted plasma on cellulose acetate membrane. Barbital buffer pH 8.6 and ionic strength 0.05. Applied current 3.0 ma for 30 minutes. The buffers used for changing the pH values of plasma proteins from top to bottom are phosphate pH 7.0, acetate pH 4.2, carbonate pH 10.8 and 10.0, acetate pH 5.0, phosphate pH 6.0, barbital pH 7.8 and 9.0.

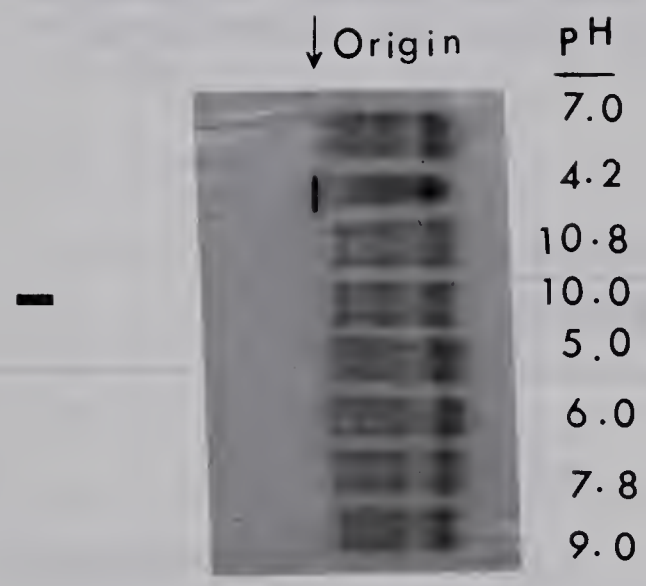

Origin
pH
7.0
4.2
10·8
10.0
5.0
6.0
7·8
9.0
−
+

Table VII. Acetylation effect on the plasma protein-bound inorganic iodide in *Esox lucius*, *Coregonus clupeaformis* and *Salmo gairdneri*.

Species	% Plasma protein-bound inorganic iodide	
	Before acetylation	After acetylation
Esox lucius	76.09	0
Coregonus clupeaformis	65.21	0
Salmo gairdneri	83.83	0

Stizostedion viterum and *Lota lota*) and bovine plasma were separated by microzone electrophoresis on cellulose acetate membrane in barbital buffer, pH = 8.6 and ionic strength 0.05. The patterns are shown in Fig. 4. While it is more difficult to achieve good separation of the plasma proteins of fish than of other animals, each fish species shows a unique electrophoretic pattern. This is one of the species specific characteristics (Deutsch and Goodloe, 1945). In *E. lucius*, *C. clupeaformis* and *S. gairdneri*, but not in the other three species of teleosts, there was a distinct protein fraction which separated from the rest and moved toward the anode with a mobility similar to that of bovine plasma albumin. It was proved that this fast migrating protein is the fraction associated with iodide binding (Fig. 12, 13, 14). These three species of fish are in the Order Clupeiformes. From these observations, it is concluded that these related species of teleosts have recognizable similarities in plasma protein fractions which are associated with unique physiological characteristics.

2. *Distribution of radioiodide on the electrophoresis membrane*

It is possible that the electrophoretic technique may disturb the true pattern of distribution of radioiodide on the plasma proteins. Marshall and Levy (1966) argued that if the strength of electric field applied was stronger than the binding energy between the thyroxine and its binding protein, the thyroxine molecule could be stripped away from the protein. The same could apply for the iodide-protein

Figure 12. A. Electrophoresis of *Esox lucius* plasma containing 0.005 ml $Na^{125}I$ (0.5 µc). The arrow indicates the origin. The peak corresponding to plasma albumin-bound inorganic iodide is to the right and those for free iodide are to the left toward the anode. Applied current 3.0 ma and duration time 30 minutes.

B. Electrophoresis of $Na^{125}I$ solution under same experimental conditions as above.

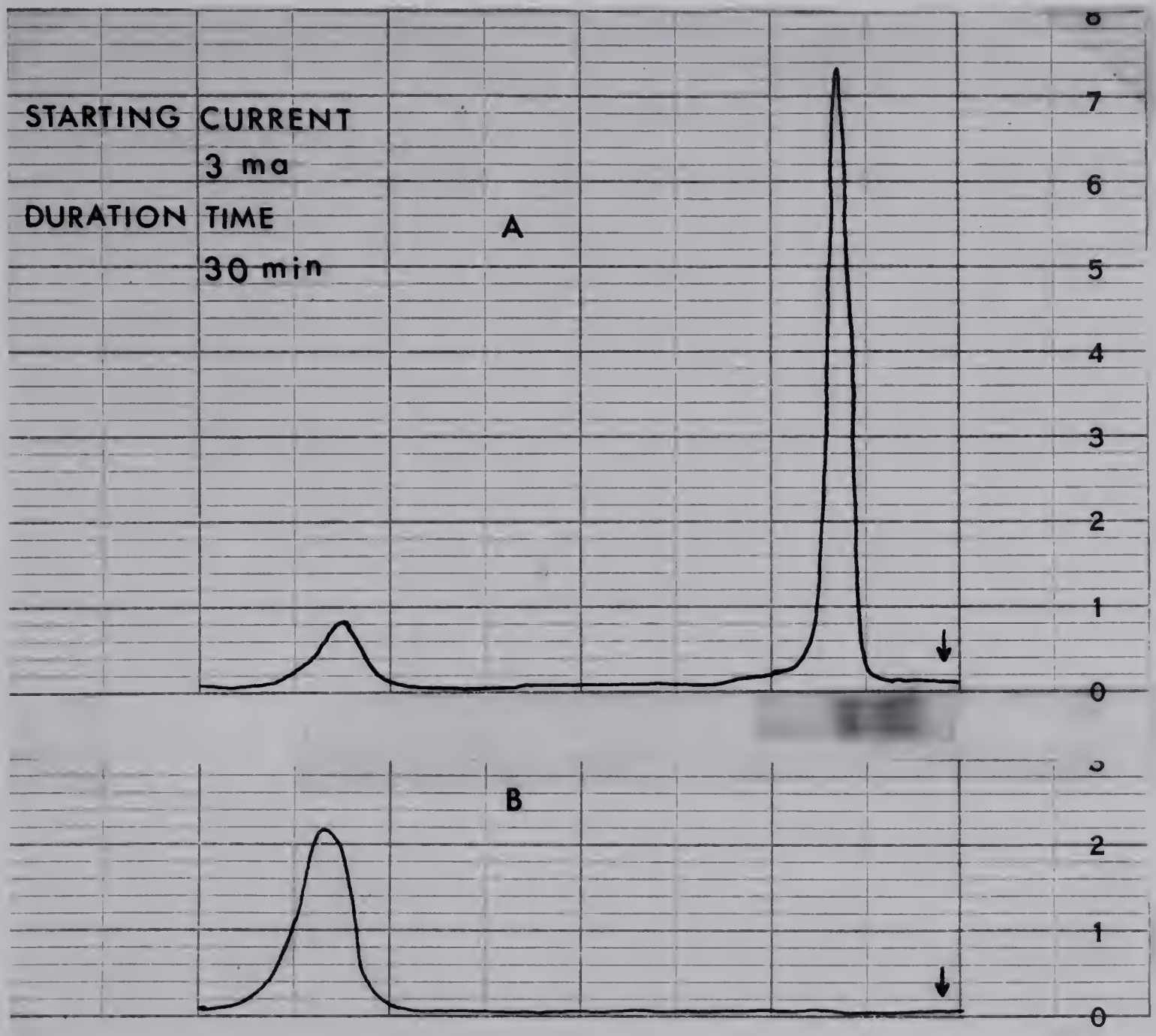

STARTING CURRENT
3 ma
DURATION TIME
30 min
A
B
7
6
5
4
3
2
1
0
2
1
0

Figure 13. Radioactive scan of *Coregonus clupeaformis* plasma containing 0.005 ml $Na^{125}I$ (0.5 µc) accompanied by an electrophoresis, showing the decrease in protein binding of iodide as the current is increased. The arrows indicate the origin. The anode is to the left.

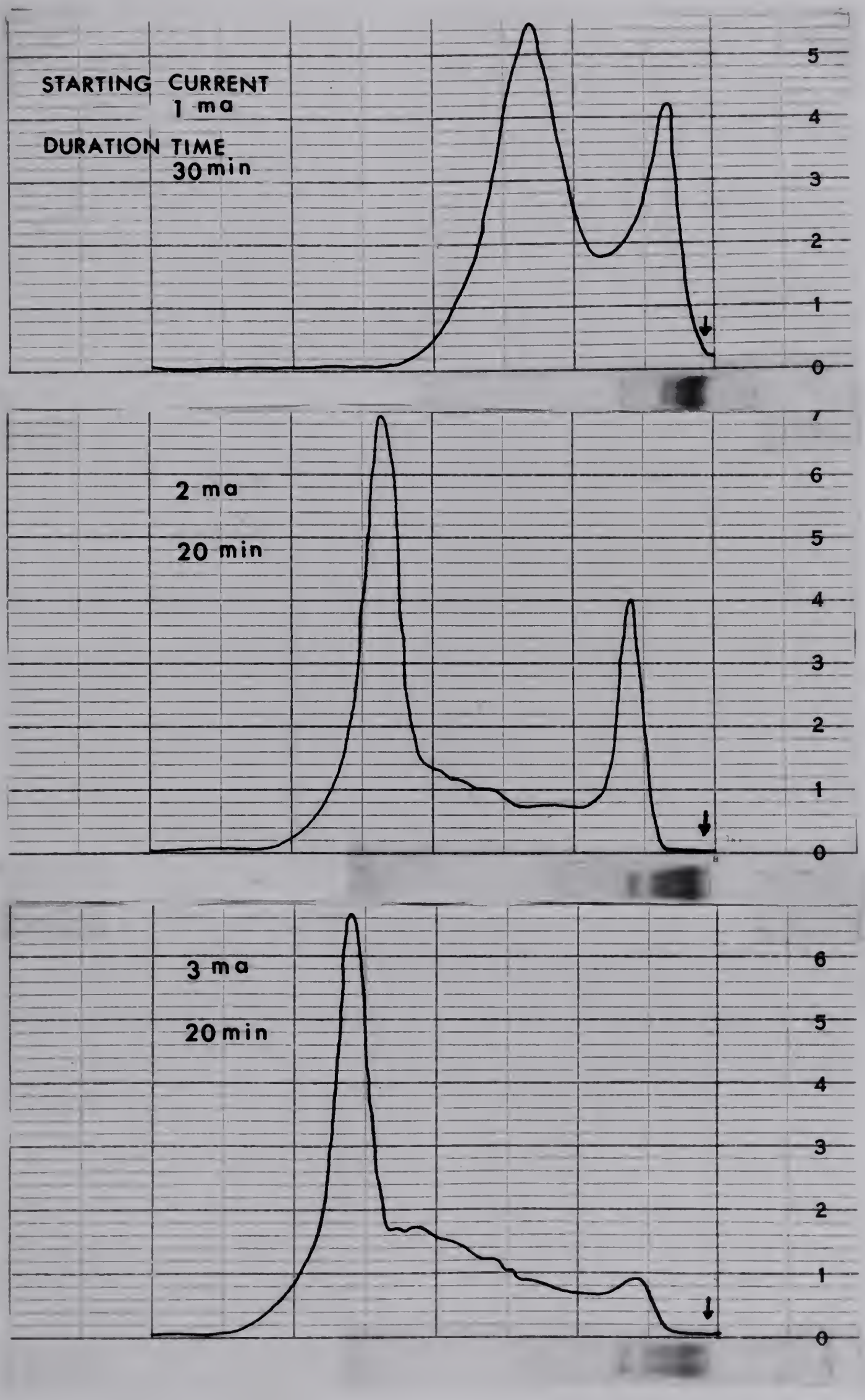
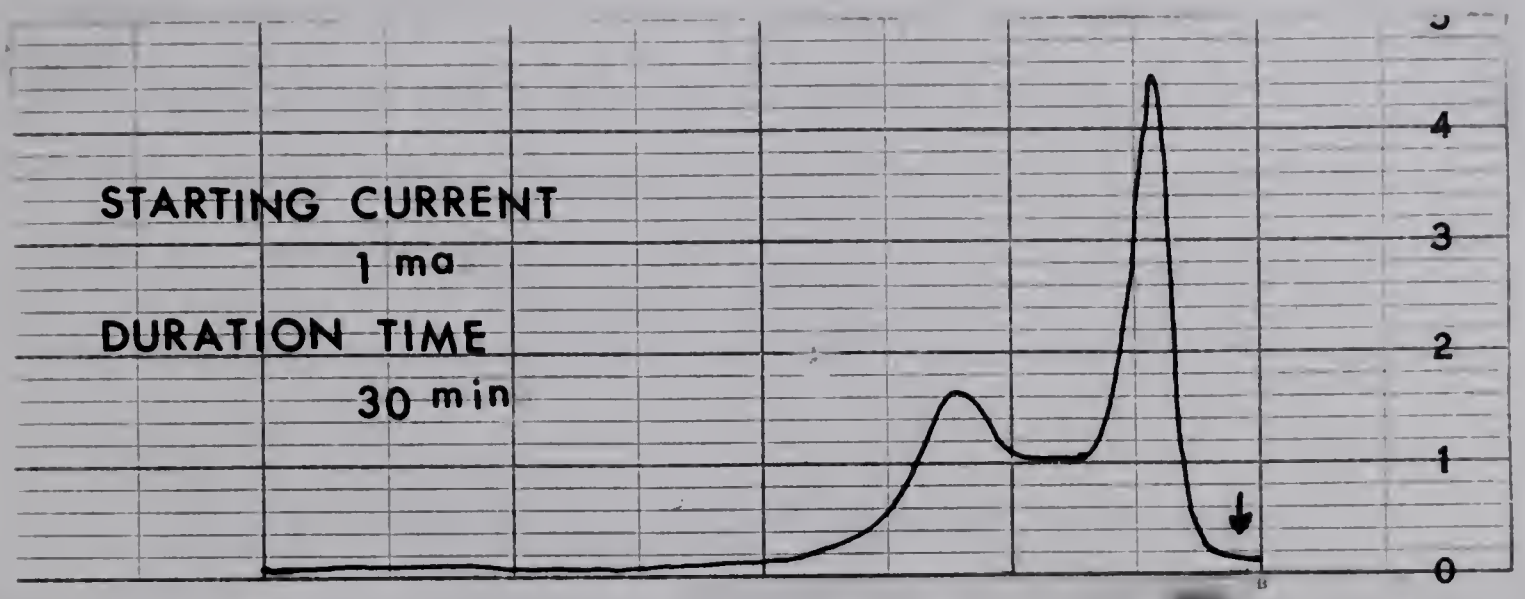
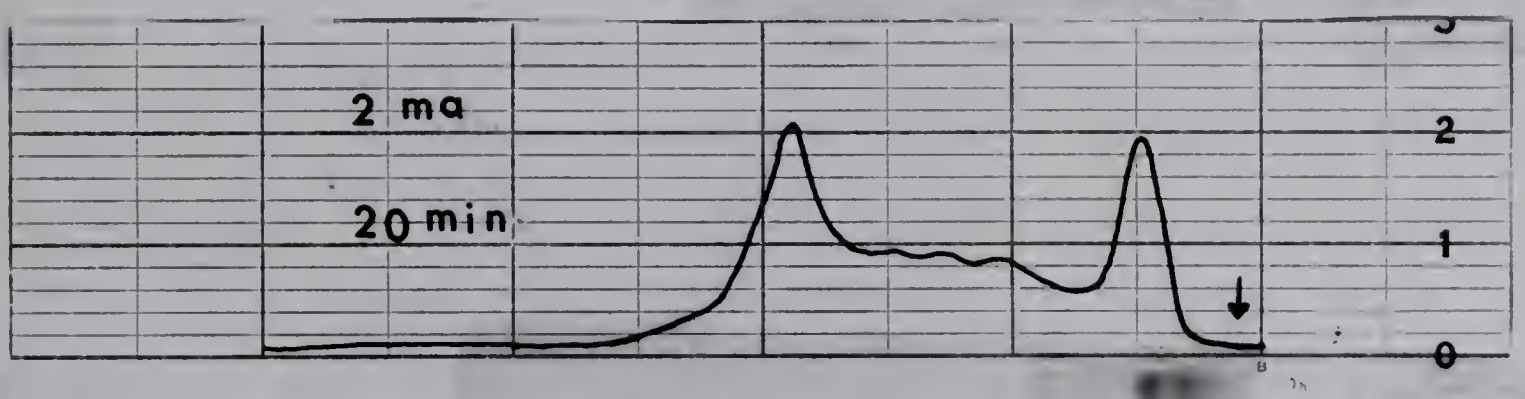
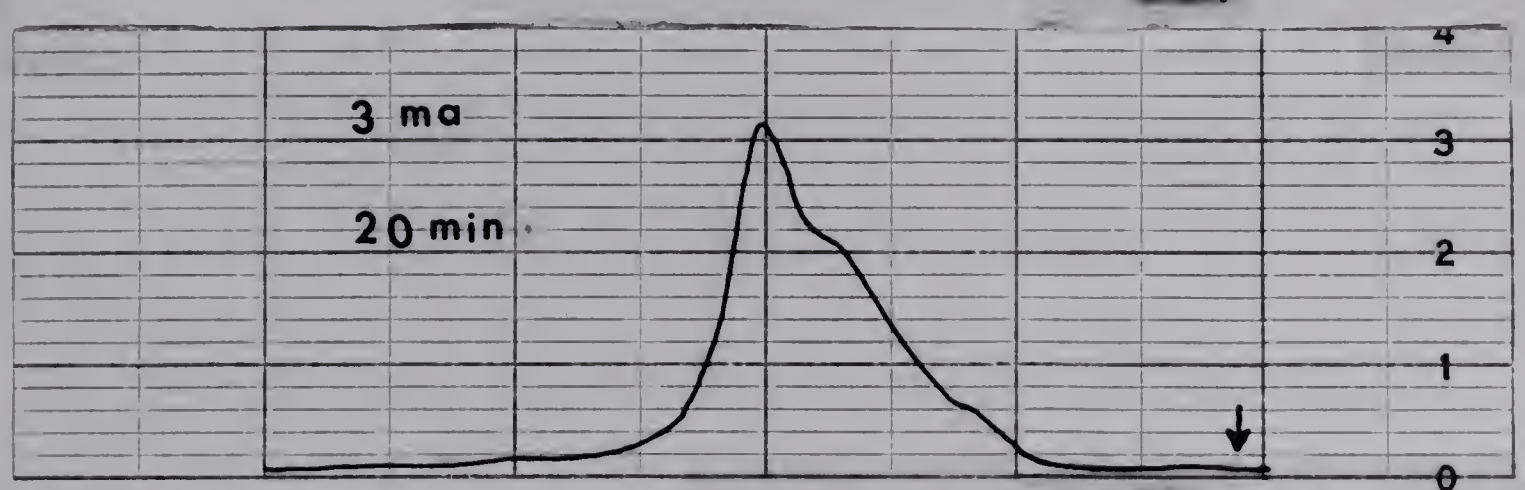

STARTING CURRENT
1 ma
DURATION TIME
30 min
5
4
3
2
1
0
2 ma
20 min
6
5
4
3
2
1
0
3 ma
20 min
6
5
4
3
2
1
0

Figure 14. Radioactive scan of *Salmo gairdneri* plasma containing 0.005 ml $Na^{125}I$ (0.5 µc) accompanied by an electrophoresis, showing the decrease in protein binding of iodide as the current is increased. The arrows indicate the origin. The anode is to the left.


STARTING CURRENT
1 ma
DURATION TIME
30 min
4
3
2
1
0


2 ma
20 min
2
1
0


3 ma
20 min
4
3
2
1
0

interaction. In addition, the time needed for separation and the heat produced during electrophoresis might also interfere with the radioactivity distribution on the membrane. For the last mentioned reason, electrophoresis was always carried out at 4°C to limit heat production and minimize disturbance of the association of radioiodide with the binding protein.

Figure 12 shows the plasma protein separation of *E. lucius* at a starting current of 3.0 ma and duration time of 30 minutes, the same conditions used for normal plasma proteins separation. Comparison of the radioactivity distribution with the electrophoretic pattern of plasma proteins (Fig. 12 A) showed that there were two radioactive peaks present: one sharp peak located on the albumin-like fraction and another much smaller peak running far in advance of the protein. This small peak coincided with migration of the control $Na^{125}I$ solution (Fig. 12 B) and was therefore free iodide.

The strength of applied current and the duration of electrophoresis have a very significant effect on the protein-iodide interaction in the plasma of *C. clupeaformis* and *S. gairdneri* (Fig. 13 and 14). As the graphs show, binding is significantly reduced on the albumin-like fraction as the applied current is increased. It virtually disappears at a current of about 3.0 ma for 20 minutes. However, 2 radioactive peaks were evident under the experimental conditions of 2.0 ma for 20 minutes and 1.0 ma for 30 minutes. For these

species, the conditions of 2.0 ma and 20 minutes gave good results and radioactivity distributions that were nearly the same as for *E. lucius*. These results suggested the binding energy between the ^{125}I and plasma albumin-like protein of *C. clupeaformis* and *S. gairdneri* may be weaker than between those of *E. lucius*. However, other aspects of the protein molecular structures must be considered in arriving at an explanation for the species difference.

C. Separation of the Plasma Proteins by Precipitation Methods

Trichloroacetic acid precipitated all of the plasma proteins and totally destroyed its iodide binding capacity (Table VIII). The results, which confirm those of Leloup and Fontaine (1960), are interpreted to mean that trichloroacetic ions are more easily bound to the plasma proteins than iodide (Scatchard *et al.*, 1957).

Half-saturated ammonium sulfate precipitation of the plasma globulins provided additional evidence that the iodide was bound to the albumin in these three species of fish. As shown in Table IX, in each case the radioactivity was confined almost entirely to the supernatants, which included the albumin, after the precipitation of the globulins.

Table VIII. Trichloroacetic acid (20% solution) precipitation of the plasma proteins and the resultant destruction of the inorganic iodide binding capacity in *Esox lucius*, *Coregonus clupeaformis* and *Salmo gairdneri*.

Species	% Plasma proteins-bound inorganic iodide (Equation 8)	
	Before TCA precipitation	After TCA precipitation
Esox lucius	75.42	0.77
Coregonus clupeaformis	77.55	1.88
Salmo gairdneri	83.83	1.88

Table IX. Half-saturated ammonium sulfate precipitation of the globulins in the plasma of *Esox lucius*, *Coregonus clupeaformis* and *Salmo gairdneri*, showing that the radio-iodide resides in the supernatant, containing the albumin fraction.

	% Distribution of ^{125}I in the plasma proteins (Equation 8)	
Species	Supernatants (albumin *et al.*)	Precipitates (globulins)
Esox lucius	98.33	1.67
Coregonus clupeaformis	97.63	2.37
Salmo gairdneri	98.00	2.00

DISCUSSION

A. Extent of the Binding of Inorganic Iodide With Plasma Protein in Certain Teleosts

Of eight species of non-migratory freshwater teleosts investigated in this study, the binding of inorganic iodide with plasma protein existed only in species of the Order Clupeiformes: *E. lucius*; *C. clupeaformis*; *S. gairdneri* and *T. arcticus*. The binding characteristic was not present in the other Orders studied. Leloup and Fontaine (1960) and Leloup (1964), stated that this physiological characteristic was (1) present only in the teleost fish among all vertebrates, (2) associated with neither the habitat (fresh or salt water) nor the migratory habit of teleosts and (3) restricted to the Orders Clupeiformes and Mugiliformes of the teleost fishes.

B. Seasonal Variation of Iodide Binding Capacity in Lake Fish

It was shown that the overall percentage changes in iodide binding capacity with season were small. However, it is significant that (1) both sexes show similar variation seasonally and (2) male fish have a higher binding capacity than females in *E. lucius* and *C. clupeaformis* (Fig. 5 and 6; Table II and III). In searching for an explanation for the seasonal change, two parameters, the plasma albumin-like protein concentration and the plasma inorganic iodide content were considered because change in either might influence iodide binding.

1. *Variation of plasma albumin-like protein*

There is abundant evidence that the quantity and pattern of the plasma proteins of fish are affected by physiological and environmental changes. Leloup and Fontaine (1960) reported changes in the blood proteins corresponding to gonad maturation and migration of Atlantic salmon, *Salmo salar*. Robertson *et al.*, (1961) found sex differences in total protein, albumin and globulin concentration and a decrease in concentrations of plasma total proteins, globulins and albumins during the anadromous migration of Pacific salmon, *Oncorhynchus tshawytascha*. Vanstone and Ho (1961) also found sex and age differences in total plasma proteins and in protein fractions in Coho salmon, *Oncorhynchus kisutch*. Sano (1960a and b) noted a seasonal variation of total serum proteins and variations in protein concentration with growth of rainbow trout. Meisner and Hickman (1962) found the albumin/globulin ratio of rainbow trout, *S. gairdneri*, to vary with the acclimation temperature. Booke (1965) reported an increase in the serum globulin level with age in lake whitefish, *C. clupeaformis*. In a literature review of the subject (Booke, 1964a), concluded that sex, spawning, food, osmotic pressure, temperature, light, age, "hibernation", hormones, oxygen depletion and season were factors which have been shown to affect the serum protein components of fish.

In the present study, the plasma albumin-like protein fraction was shown to possess the iodide binding capacity

(Fig. 12, 13 and 14). In both *C. clupeaformis* and *E. lucius*, male fish had a higher plasma albumin-like protein level than females, but the difference was not statistically significant (Table III). Furthermore, the iodide binding capacity of the plasma albumin-like protein was independent of albumin concentration in both sexes of the two species studied (Table IV). Klotz (1950) notes that a five-fold (0.2 - 1.0g%) increase in bovine plasma albumin concentration produced only a very small change in the extent of binding of methyl orange. Karush and Sonenberg (1949) also noted no detectable change in the extent of alkyl sulfate binding even with a ten-fold (0.05 - 0.5 g%) change in plasma albumin. These reports suggest, then, that the direct effect of protein concentration on its ion binding capacity is small, and therefore supports a similar observation in the present study. However, many other factors, such as the differences in plasma pH, in plasma dielectric constant, and in plasma protein structure might change the total available binding sites on the protein. Any of these factors might cause the observed sex difference in binding.

2. *Variation of plasma iodide content*

It seems probable that any of several environmental factors (water iodide content, water temperature and photoperiod) and physiological status of fish can cause variation in plasma iodide content. Hickman (1962) demonstrated a seasonal change in plasma iodide concentration in *C. clupeaformis* which corresponded roughly to the water temperature.

He suggested that the equilibrium state of iodide was influenced by environmental temperature acting through the metabolic rate of the fish: with increasing water temperature, the rate of iodide uptake slightly exceeded its loss from the body. As a result the plasma iodide content was high in summer. With falling water temperature in the autumn, iodide excretion exceeded its intake, and a drop in the plasma iodide resulted. Hickman (1962) suggested that iodide was actively absorbed from the water by the gills. Two probable consequences of this iodide binding are: (1) to reduce the renal and branchial excretion of plasma iodide and (2) to reduce the rate of penetration of plasma iodide into other tissues (Leloup and Fontaine, 1960; Jacoby, 1965). This suggests that, in addition to an increase in the rate of iodide uptake, an increase in iodide binding capacity may occur in summer which causes an increase in plasma iodide content. However, seasonal dietary changes, or factors other than water temperature, or binding capacity, could be responsible for this fluctuation.

Variation of iodide binding capacity has been studied in Atlantic salmon, *Salmo salar* and rainbow trout, *Salmo gairdneri* by Leloup and Fontaine (1960). They found that variations were related to different stages of the life cycle. Binding capacity decreased as the ovary weight increased and was lowest in sexually mature fish. In conjunction with this, they found that the stable iodide content in the ovaries of salmon and trout was about 10 times

its concentration in plasma. Developing ovaries are known to accumulate large amounts of iodide and at maturity the iodide content in the ovary may exceed that of all the rest of the body tissues combined (Robertson *et al.*, 1953; Leloup and Fontaine, 1960; Lindsay *et al.*, 1966). In this study, as illustrated in Fig. 5 and 6, a rough inverse relationship existed between the iodide binding capacity and the gonad (especial ovary) maturation in both *E. lucius* and *C. clupeaformis*. However, the physiological purpose (or possible adaptive advantage) of the decrease in iodide binding with gonad maturation is unknown.

Leloup and Fontaine (1960) have compared the iodide binding capacity of the plasma proteins and the plasma iodide content among the vertebrates. They found that the plasma iodide level in cyclostomes, lung fish, birds and mammals is much lower, as a rule, than that of anadromous teleosts. They hypothesized that the binding of iodide with the plasma protein is an important factor in maintaining a higher level of plasma iodide needed for thyroid hormones synthesis by those anadromous teleosts. In this study, the iodide binding species (*E. lucius*, *C. clupeaformis*, and *S. gairdneri*) show a relative greater plasma iodide level than do those species without this binding characteristic (*C. commersonii* and *S. vitreum*) (Hickman, 1962 and unpublished data; McNabb, 1963). However, the differences in plasma iodide content in the teleosts presumably are not related entirely to their iodide binding characteristic. Other factors, such as the role of active influx of iodide from the water by the gills

must be considered (Hickman, 1962). It also suggests that the iodide binding characteristic of these fish species is not necessarily related to thyroid hormones synthesis for higher metabolic activity, since the species studied are non-migratory forms and are not appreciably more active than species not having the binding characteristic.

C. Nature of Inorganic Iodide Binding Process

The interaction of inorganic ions with proteins, especially anions with isolated mammalian serum albumin, has been extensively investigated. A general hypothesis to explain the interaction of anionic dyes and thyroxine with cationic groups of basic amino acid residues (e.g. lysine, arginine and histidine) on the plasma protein molecules has been provided by Klotz *et al* (1946) and Sterling *et al*., (1962). The combination results from the electrostatic attraction between the two oppositely charged groups. In this study, observations have been extended to consider the effect on the iodide-protein interaction of variations in temperature, pH, acetylated protein and the addition of other monovalent anions and antithyroid substances to the dialysis equilibrium system. The results further support the concept that inorganic iodide is bound to the free cationic groups of basic amino acid residues on the plasma albumin-like protein molecules. These factors will be discussed in turn.

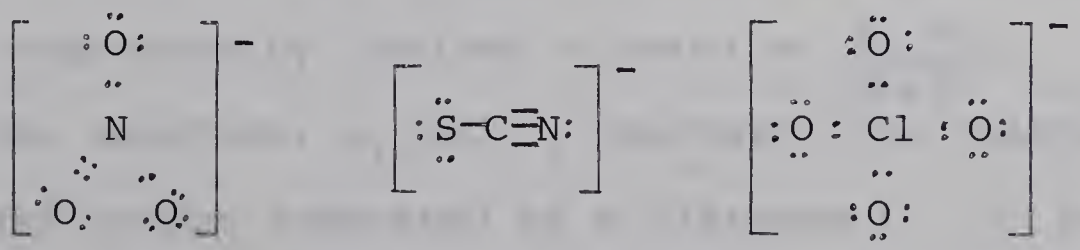
1. *Effect of pH, buffers and acetylation on the plasma protein binding characteristic*

Klotz and Urquhart (1949a) compared the relative binding affinities of various buffer anions with bovine serum albumin by studying their competition between anions and anionic dyes. They found the relative binding affinity to be of the order glycinate < phosphate < bicarbonate < acetate < chloride < carbonate < barbital < citrate < nitrate < phthalate. Four buffers, acetate, phosphate, barbital and carbonate were employed in this study. The direct competition between buffer anions and iodide for loci on the plasma albumin-like protein presumably is small, because the affinity of iodide with the plasma protein is stronger than that of the buffer anions. As illustrated in Fig. 10 *E. lucius* plasma albumin-like protein shows its highest binding capacity at pH 7.0, since, in the neutral solution, the iodide can freely combine with the cationic groups (the free $-NH_3^+$ group) of the protein molecules. In more acidic solution, more hydrogen ions associate with the iodide (bond energy about -71.4 Kcal/mole) (Hiller and Herber, 1960) and, as a result, progressively displace the iodide ions from the plasma protein. In more basic solutions, there is also a distinct drop in binding capacity, leading finally to complete loss of binding capacity at pH 10.8. In this case, the reduction of iodide binding is due primarily to the loss of protons from the cationic groups, especially the free $-NH_3^+$ groups of lysine residues. Additional evidence for this view

is afforded by the observation that binding was diminished after lysyl ε-NH_3^+ groups of the plasma proteins in *E. lucius*, *C. clupeaformis* and *S. gairdneri* were blocked specifically by acetylation (Table VII). These two experimental evidences strongly suggest that the free-NH_3^+ groups are the major binding sites for iodide.

2. *Nature of iodide and other monovalent anions*

Figure 9 showed that the formation of the ^{125}I-protein complex was inhibited by the addition of NO_3^-, SCN^-, $^{127}I^-$ and ClO_4^- but not affected by the F^-, Cl^-, Br^- in the three fish species studied. This suggests that the iodide-protein interaction, like most anion-protein interactions, is non-specific in nature. Differences in affinity with the protein depends on the nature of the anion. F^-, Cl^- and Br^- are more strongly bound to Na^+ than with I^- in the solution. As a result, they did not significantly interfere with the ^{125}I-plasma protein interaction. The effect of stable iodide on the ^{125}I binding, which is distinct from that of NO_3^-, SCN^- and ClO_4^-, will be discussed in the next section. The important point to consider here is the ionic structure of the polyatomic anions, NO_3^-, SCN^- and ClO_4^-. The electron dot formula for these anions are (Hiller, and Herber 1960):



The NO_3^- is stabilized by resonance structures in solution. All three polyatomic anions have extra free electron pairs which are available to the cationic groups of the plasma protein molecules. These polyatomic anions inhibited the formation of ^{125}I-protein complex through competition with ^{125}I ions for the same binding sites on the plasma protein molecules. Leloup (1964b) suggested a similar competitive effect. Carr (1952),using a protamine-collodion exchange membrane, found the binding affinity of anions with serum albumin in the order $Cl^- < Br^- < NO_3^- < I^- = SCN^-$. Scatchard and co-workers (1957) also compared the anion binding with plasma albumin by using an electrode system involving an anion-exchange membrane. They found the binding affinity to increase in the order $Cl^- < I^- < SCN^- < CCl_3COO^-$. In general, the binding of the anions is assumed to occur at the same sites on the protein molecules. Klotz (1950) attributed the greater binding affinity of certain anions to protein molecules to their greater ability to release water molecules from the ion-protein complex, rather than to an increase in Van der Waal forces which accompany increased molecular size of the ion. The binding forces between cationic groups of protein molecules and anions are presumably electrostatic in nature. This electrostatic force may be quantitatively expressed by Coulumb's Law: $F = \frac{e_1 e_2}{\epsilon r^2}$.

In the equation, e_1 and e_2 represent the charges on two charged groups separated by a distance r. ϵ is the dielectric constant of the medium. Protein molecules and inorganic iodide in plasma are surrounded by water molecules.

The removal of water molecules from the iodide-protein complex causes an increase in the electrostatic force, since it reduces the distance (r) between these two charged groups. The dielectric constant (ϵ) of the plasma is decreased also by increasing the free water molecules in the medium. The destruction of the iodide binding capacity of plasma protein by TCA precipitation (Table VIII) also suggests that the decrease in plasma dielectric constant is caused by adding this organic acid solution (lower dielectric constant). Consequently the electrostatic forces between CCl_3COO^- and cationic groups of plasma protein molecules as well as the charged groups of the same or neighboring protein molecules is increased (Pennell, 1960).

Thiourea and thiouracil are known to chemically reduce free iodine with great rapidity *in vitro* (Carr and Riggs, 1958). Neither substance had any effect on the iodide-plasma protein interaction in the three fishes studied (Fig. 9). This suggests that inorganic iodine exists in the plasma entirely as the iodide ion and therefore does not react with these reducing substances. Leloup (1964) had arrived at the same conclusion.

3. *Binding energy and percentage of binding in iodide-plasma protein interaction*

The energy of binding and the percentage of binding in most ion-protein interactions studied are not the same, since these interactions are temperature independent (Davis, 1943; Putnam and Neurath, 1945; Boyer, Ballou and Luck, 1947;

Klotz and Curme, 1948; Klotz and Urquhart, 1949b; Karush and Sonenberg, 1949; Scatchard, Scheinberg and Armstrong, 1949). Let us consider the thermodynamic equation:

$$\Delta F = \Delta H - T\Delta S.$$

If the ion-protein interaction is temperature independent in nature, the ΔH (heat of reaction or enthalpy change) must be small, and the magnitude of ΔF (free energy or binding energy) at any given temperature (T) is determined primarily by the value of ΔS (the entropy change in the reaction). Many ion-protein interactions were shown to have a greater entropy (ΔS) change than heat effect (ΔH) during the binding process (Klotz, 1950). In the present study (Table V and VI), the per cent of iodide binding was shown to be significantly higher at 4°C than at 20°C for *E. lucius* and *C. clupeaformis*. This difference is related to the association constant of their iodide-protein complexes (0.42×10^5 liter/mole at 4°C and 0.310×10^5 liter/mole at 20°C for *E. lucius* and 0.81×10^5 liter/mole at 4°C and 0.25×10^5 liter/mole at 20°C for *C. clupeaformis*). Leloup (1964b) found that the per cent of free iodide varied in Atlantic salmon from 4.7% at 4°C to 8.0% at 17°C with association constants of 0.65×10^6 liter/mole and 0.35×10^6 liter/mole respectively.

The binding energy for the association constant was calculated by the equation:

$$\Delta F^\circ = -RT \ln K \quad - - - - - - - - - - - - - (9)$$

where ΔF° represents the change in standard free energy or the binding energy of the complex. R is the gas law constant,

T is the absolute temperature and K is the association constant. Table VI showed that there was no appreciable difference in binding energy of the iodide plasma protein complex at two experimental temperatures (-5.898 Kcal/mole at 4°C and -6.054 Kcal/mole at 20°C for *E. lucius* and -6.252 Kcal/mole at 4°C and -5.903 Kcal/mole at 20°C for *C. clupeaformis*). We have calculated the binding energy of salmon from Leloup's data: -7.403 Kcal/mole at 4°C and -7.395 Kcal/mole at 17°C. These data show that the iodide-plasma protein interactions of the fish studied are all temperature independent in nature. However, binding capacity increases at lower temperatures because of the increase in the association constant.

Figure 8 showed that the percentage of ^{125}I binding was decreased by increasing the total concentration of stable iodide in the plasma. Since radioactive ^{125}I and stable ^{127}I have nearly identical physical-chemical properties, the distribution of ^{125}I in the dialysis system represents the distribution of stable iodine in the same system. The total inorganic iodine concentration of *E.lucius* plasma was 2.567 µg% (0.202×10^{-6} M). Since about 87.85% of the total plasma iodide was protein bound at 4°C, the value of the bound iodide fraction was 0.177×10^{-6} M and of the free iodide, 0.025×10^{-6} M. When 0.67×10^{-2} M of stable iodide was added to the plasma, the per cent of bound iodide was dropped to 71.25%, but both the bound iodide and free iodide concentrations were increased to 40.31×10^{-6} M and 16.27×10^{-6} M respectively in the dialysis system. When 2.68×10^{-2} M

of stable iodide was added to the plasma, the per cent of bound iodide was dropped to 49.83%, but the concentrations of bound iodide and free iodide were increased to 71.44 x 10^{-6} M and 71.93 x 10^{-6} M respectively in the dialysis system. Similar results were obtained with *C. clupeaformis* plasma. In summary, the overall effect of increasing the total iodide concentration in the plasma was to increase the concentration of bound iodide while decreasing the percentage of bound iodide. The decrease in per cent binding with increasing iodide concentration was caused by: (1) increasing electrostatic repulsion between like anions (^{125}I and ^{127}I) and (2) reducing the available binding sites on the plasma protein molecules.

4. *Some remarks on the molecular structure of the plasma protein*

In addition to the nature of ions, the molecular structure of protein is important for the formation of the ion-protein complex. The kind and sequence of amino acids in the polypeptide chain, the number of polypeptide chains, the hydrogen bonding formation and the helical structure of the protein are the important parameters of its structure. The structure of human and bovine plasma albumin is much better known than that of other animal species. There is a general agreement that both human and bovine plasma albumin have a single polypeptide chain, and are similar in molecular size and general amino acid composition (Putnam, 1960). The bovine plasma albumin molecule also showed about 46% of α-helical

structure (Urnes and Doty, 1961). Leloup (1960) noted that the molecular weight of plasma albumin-iodide complex of *Salmo salar* was about 70,000.

Klotz (1950) compared the amino acid composition with the binding ability of several proteins, and suggested a "binding index" for the ion-protein complex formation. This binding index $\frac{\Sigma(\equiv NH^+)}{|\ \Sigma(-OH) - \Sigma(-COO^-)\ |}$ is based on the relative numbers in a protein of basic amino acids (lysine, arginine and histidine), acidic amino acids (aspartic acid and glutamic acid) and amino acids with free hydroxyl groups (serine, threonine, tyrosine and hydroxyproline). Three types of hydrogen bonds are formed between these polar groups with different binding energies in the order COO^--OH (6 Kcal/mole) > COO^- -NH_3^+ (3.5 Kcal/mole) > NH_3^+ -OH (2.5 Kcal/mole) (Pauling, 1945; Davis, 1946; Pressman, Grossberg, Pence and Pauling, 1946). Klotz (1950) thought that if the number of either one of two functional groups (hydroxyl and carboxyl groups) exceeded that of the other, the excess number of residues would be available to combine with the cationic nitrogen ($\equiv NH^+$) and thereby to decrease the ability of the proteins to bind anions. Based on this formula, he found bovine plasma albumin to have the highest binding index of any proteins studied. This index was compatible with his experimental results.

Many other theories about the anion protein interaction have appeared in the literature. Schellman (1953) stated that the anion binding was based on partial shielding of

the ion pairs from the polar solvent. Saroff (1956) developed a theory of chloride binding for plasma albumin in terms of hydrogen binding. Groth, Kunze and Segal (1963) suggested that, on the basis of stereochemical considerations, both cations and anions may bind into certain hydrogen bridges of the non-helical parts of globular protein molecules.

In summary, this research has demonstrated that the iodide-plasma albumin-like protein interaction of the teleost species studied is non-specific in nature, is temperature independent and is weak in binding energy. This suggests that the interaction is a hydrogen bond or an electrostatic force. However, further studies on the molecular structure of the plasma proteins of teleost fish are required to understand the marked species specificity of this characteristic and its possible adaptive value.

SUMMARY

1. The binding of inorganic iodide to plasma protein of normal plasma was demonstrated in species of the Order Clupeiformes, including northern pike, *Esox lucius*; lake whitefish, *Coregonus clupeaformis*; rainbow trout, *Salmo gairdneri*, and arctic grayling, *Thymallus arcticus*. This characteristic did not exist in white sucker, *Catostomus commersonii*; yellow perch, *Perca flavescens*; yellow walleye, *Stizostedion vitreum* and burbot, *Lota lota*. These last four species belong to other teleost Orders.

2. Plasma inorganic iodine is present as iodide and is bound with the plasma albumin-like protein of the teleost species studied. The actual binding sites are presumably the free cationic groups of basic amino acid residues such as lysine, arginine and histidine on the plasma albumin-like protein molecules.

3. Small seasonal variations in the plasma iodide binding capacity were found and were of the same pattern in both sexes of *E. lucius* and *C. clupeaformis*. The males had a significantly higher binding capacity than females in these two species.

4. Seasonal variation in the plasma iodide binding capacity of some fish species is probably one of the factors causing seasonal fluctuations in plasma iodide content.

Binding capacity is evidently independent of the plasma albumin concentration.

5. The binding is non-specific in nature. It can be inhibited by NO_3^-, SCN^- and ClO_4^-, probably through competition for binding sites. It is not affected by thiourea, thiouracil, F^-, Cl^- and Br^-.

6. The binding capacity can be completely destroyed by trichloroacetic acid precipitation of the plasma proteins.

7. The binding energy is temperature independent. The binding energy is weak in *E. lucius* (-5.897 Kcal/mole at 4°C) and *C. clupeaformis* (-6.252 Kcal/mole at 4°C).

8. The per cent of bound iodide is related to the association constant. Both are inversely related to the temperature. The per cent of bound iodide at 20°C and 4°C is, for *E.lucius* 80.85% (20°C), 88.85% (4°C); for *C. clupeaformis* 80.28% (20°C), 88.75% (4°C); and for *S. gairdneri* 84.30% (20°C), 92.23% (4°C). In *T. arcticus*, the per cent of bound iodide is 80.97% at 26°C.

9. The per cent of bound iodide is decreased by the addition of stable iodide to the plasma, but the absolute concentration of bound iodide is increased.

10. The iodide-plasma protein interaction is presumably a hydrogen bonding or an electrostatic force between the iodide and the cationic groups of the plasma protein molecules.

LITERATURE CITED

Acland, J. D. 1957. The estimation of serum protein-bound iodide by alkaline incineration. *J. Biochem.* 66:177-188.

Booke, H. E. 1964a. A review of variations found in fish serum proteins. *New York Fish Game J.* 11:47-57.

Booke, H. E. 1965. Increase of serum globulin levels with age in lake whitefish, *Coregonus clupeaformis*. Trans. of Amer. Fish. Soc. (T.A.F.S.). 94:347-398.

Booth, M., Dixon, P. F., Gray, C. H., Greenaway, J. M., and Holness, N. J. 1961. Protein binding of cortisal in health and in pregnancy. *J. Endocrinol.* 23:25-35.

Boyer, P. D., Balou, G. A., and Luck, J. M. 1947. The combination of fatty acids and related compounds with serum albumin. III. The nature and extent of the combination. *J. Biol. Chem.* 167:407-424.

Carr, C. W. 1952. Studies on the binding of small ions in proteins solutions with the use of membrane electrodes. I. The binding of chloride ion and other inorganic anions in solution of serum albumin. *Arch. Biochem. Biophys.* 40:286-294.

Carr, E. A. and Riggs, D. S. 1958. Thyroid gland. In: *Pharmacology in Medicine* 2nd ed. (V. A. Drill, ed.) Chap. 71, pp. 1021. McGraw-Hill Book Co. Inc., New York.

Davis, B. D. 1943. The binding of sulfonamide drugs by plasma proteins. A factor in determining the distribution of drugs in the body. *J. Clin. Invest.* 22:753-762.

Deutsch, H. F. and Goodloe, M. B. 1945. An electrophoretic survey of various animal plasmas. *J. Biol. Chem.* 161:1-20.

Foster, J. E. 1960. Plasma albumin. In: *The Plasma Proteins* (F. W. Putnam, ed.). Chap. 6. pp. 179-233. Academic Press Inc., New York.

Fraenkel-Conrat, H., Bean, R. S., and Lineweaver, H. 1949. Essential groups for the interaction of ovomucoid (egg white trypsin inhibitor) and trypsin, and for tryptic activity. *J. Biol. Chem.* 177:385-403.

Groth, J., Kunze, H., and Segal, J. 1965. The interaction between globular proteins and monovalent ions. In: *Protides of the Biological Fluids* (H. Petters, Ed.). pp. 429-433. Elsevier Publishing Co., Amsterdam. London. New York.

Gurd, F. R. N., and Wilcox, P. E. 1956. Complex formation between metallic cations and proteins, peptides and amino acids. *Advances in Protein Chem.* 11:311-427.

Hickman, C. P., Jr. 1961. The conversion ratio as a discriminatory test for thyroid activity in fish. *Nature*. 189:1012-1013.

Hickman, C. P., Jr. 1962. Influence of environment on the metabolism of iodine in fish. *Gen. Comp. Endocrinol.*, Supple. 1:48-62.

Hiller, L. A. and Herber, R. H. 1960. *Principles of Chemistry*. McGraw-Hill Book Co. Inc., New York.

Ingbar, S. H., and Freinkel, N. 1960. Regulation of the peripheral metabolism of the thyroid hormones. *Recent Progr. Hormone Res.* 10:353-403.

Jacoby, G. H. 1965. A study of the circulating iodocompounds of rainbow trout, *Salmo gairdneri*. M.Sc. Thesis, Univ. of Alberta.

Karush, F., and Sonenberg, M. Interaction of homologous alkyl sulfates with bovine serum albumin. *J. Amer. Chem. Soc.* 71:1369-1376.

Klotz, I. M., Walker, F. M., and Pivan, R. B. 1946. The binding of organic ions by proteins. *J. Amer. Chem. Soc.* 68:1486-1490.

Klotz, I. M., and Curme, H. G. 1948. The thermodynamics of metallo-protein combinations. Copper with bovine serum albumin. *J. Amer. Chem. Soc.* 70:939-943.

Klotz, I. M., and Urquhart, J. M. 1949a. The binding of organic ions by proteins. Buffer effects. *J. phys. Colloid. Chem.* 53:100-114.

Klotz, I. M., and Urquhart, J. M. 1949b. The binding of organic ions by proteins. Effect of temperature. *J. Amer. Chem. Soc.* 70:847-851.

Klotz, I. M. 1950. The nature of some ion-protein complex. *Cold Spring Harber Symposia Quant. Biol.* 14:97-112.

Klotz, I. M. 1953. Protein interactions. In: *The Proteins*, 1st Edition. (H. Neurath and K. Bailey, eds.). Vol. I, Part B, Chap. 8, pp. 727-806.

Klotz, I. M. 1957. Interaction of organic molecules with proteins. In: *Molecular Structure and Biological Specificity* (L. Pauling, ed.). A.I.B.S. No. 2, pp. 91-115.

Leloup, J. and Fontaine, M. 1960. Iodine metabolism in lower vertebrates. In: *Modern Concepts of Thyroid Physiology, N. Y. Acad. Sci.* 86:316-353.

Leloup, J. 1964a. Répartition zoologique de la protéine plasmatique de liaison des iodures chez les poisson. *J. Physiol. Paris.* 56:593-594.

Leloup, J. 1964b. Caractéristiques de la liaison des iodures dans le plasma de certain Téléosteen. *J. Physiol. Paris.* 56:594-595.

Lindsay, R. H., Romine, C., Zacharewicz, F., Dupree, H. K. and Steed, K. E. 1966. Accumulation of ^{131}I by channel catfish, *Ictalurus punctatus*, ovaries *in vivo* and *in vitro*. *Gen. Comp. Endocrinol.* 6:231-238.

Long, C. 1961. *Biochemists Handbook.* E. & F. N. Spon, Ltd., London. pp. 22-42.

Marshall, J. S. and Levy, R. P. 1966. Polyacrylamide electrophoretic study of thyroxine binding to human serum. *J. Clin. Endocrinol. Metab.* 26:87-98.

McNabb, R. A. 1963. Contribution of dietary and environmental iodine to iodine balance of rainbow trout, *Salmo gairdneri*. M. Sc. Thesis, Univ. of Alberta.

Meisner, H. and Hickman, C. P., Jr. 1962. Effect of temperature and photoperiod on the serum proteins of the rainbow trout, *Salmo gairdneri*. *Canad. J. Zool.* 40(2):127-130.

Olcott, H. S. and Fraenkel-Conrat, H. 1947. Specific group reagents for proteins. *Chem. Rev.* 41:151-197.

Pennell, R. B. 1960. Fractionation and isolation of purified components by precipitation methods. In: *The plasma proteins* (F. W. Putnam, ed.). Vol. 1, Chap. 2, pp. 9-43. Academic Press Inc., New York.

Putnam, G. W. and Neurath, H. 1945. Interaction between proteins and synthetic detergents. II. Electrophoretic analysis of serum albumin-sodium dodecyl sulfate mixtures. *J. Biol. Chem.* 159:195-209.

Robbins, J. and Rall, J. E. 1957. The interaction of thyroid hormones and protein in biological fluids. *Recent progr. Hormone. Res.* 13:161-208.

Robbins, J. and Rall, J. E. 1960. Proteins associated with the thyroxine hormones. *Physiol. Rev.* 40:415-489.

Robertson, J. and Chaney, A. L. 1953. Thyroid hyperplasia and tissue iodine content in spawning rainbow trout: a comparative study of Lake Michigan and California sea-run trout. *Physiol. Zool.* 26:328-340.

Robertson, O., Krupp, M., Favour, C., Hane, S., and Thomas, S. 1961. Physiological changes occurring in the blood of the Pacific salmon, *Oncorhynchus tshawytascha* accompanying sexual maturation and spawning. *Endocrinol.* 68(5):733-746.

Rosenberg, R. M., and Klotz, I. M. 1960. Dye binding methods. In: *A laboratory Manual of Analytical Methods of Protein Chemistry, including Polypeptides.* (R. J. Block and P. Alexander, ed.). Vol. 2, p. 131. Pergamon Press, New York.

Sano, Tokuo. 1960a. Seasonal variation of blood constituents of rainbow trout. *J. Tokyo Univ. Fish.* 46:67-75.

Sano, Takuo. 1960b. Change in blood constituents with growth of rainbow trout. *J. Tokyo Univ. Fish.* 46:77-87.

Saroff, H. A. 1957. A theory for the binding of chloride ions to serum albumin based on a hydrogen bonded model. *J. Phys. Chem.* 61:1364-1368.

Scatchard, G. and Black, E. S. 1949. The effect of salts on the isoionic and isoelectric points of proteins. *J. Phys. Colloid Chem.* 53:88-89.

Scatchard, G., Scheinberg, I. H., and Armstrong, S. H. Jr. 1950. Physical chemistry of protein solutions. IV. The combination of human serum albumin with the chloride ion. *J. Amer. Chem. Soc.* 72:535-540.

Scatchard, G., Hughes, W. L., Gurd, F. R. N. and Wilcox, P. E. 1954. The interaction of proteins with small molecules and ions. In: *Chemical Specificity in Biological Interactions.* (F. R. N. Gurd, ed.), pp. 193-219. Academic Press, New York.

Scatchard, G., Coleman, J. S. and Shen, A. L. 1957. Physical chemistry of protein solutions. VII. The binding of some small anions to serum albumin. *J. Amer. Chem. Soc.* 79:12-20.

Schellman, J. A. 1953. The application of the bjerrum ion association theory to the binding of anions by proteins. *J. Phys. Chem.* 57:472-475.

Sørensen, S. P. L. 1909. Ënzymstudien. II. Uber die Messung und die Bedeutung der Wasserstoffionenkonzentration bei enzymatischen Prozessen. *Biochem. Z.* 21:131-201.

Sterling, K. and Tabachnick, M. 1961. Paper electrophoretic demonstration of thyroxine-binding prealbumin fraction in serum. *Endocrinol.* 68:1073-1075.

Sterling, K. and Rosen, P., and Tabachnick, M. 1962. Equilibrium dialysis studies of the binding of thyroxine by human serum albumin. *J. Clin. Invest.* 41:1021-1030.

Urnes, P. J. and Doty, P. 1961. Optical rotation and the conformation of polypeptides and proteins. *Advances in Protein Chem.* 16:401-544.

Vallee, B. L. 1955. Zinc and metalloenzymes. *Advances in Protein Chem.* 10:317-384.

Vanstone, W. and Ho, F. Chung-wai. 1961. Plasma proteins of Coho salmon, *Oncorhynchus kisutch*, as separated by zone electrophoresis. *J. Fish. Res. Bd. Canada.* 18(3):393-399.